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The Role of Autophagy in Hydrogen Peroxide- or Methylglyoxal-induced Cardiac Myocyte Damage

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The Role of Autophagy in Hydrogen Peroxide- or Methylglyoxal- induced Cardiac Myocyte Damage

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We, the undersigned, duly appointed committee have read and examined this manuscript and certify it is adequate in scope and quality as a thesis for this master's degree. We approve the content of the thesis to be submitted for processing and acceptance.

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ABSTRACT

Oxidative stress (e.g., increased hydrogen peroxide [H_2O_2] levels) and dicarbonyl stress (e.g. increased methylglyoxal [MG]) serve as initiators for the pathogenesis of cardiovascular diseases and diabetic complications. Increased H_2O_2-derived free radicals and MG can be highly reactive and attack cellular components resulting in cell damage and even cell death. Normally, endogenous antioxidants and glyoxalase degrade H_2O_2 and MG, respectively. Additionally, autophagy facilitates the break-down of damaged cellular components allowing the components to be recycled by the cells. Researchers are still unclear about whether autophagy exacerbates cell damage or protects cells from higher doses of H_2O_2 and MG. This study investigated cell damage and autophagy changes when rat cardiac H9c2 myoblasts were treated with H_2O_2 or MG. Moreover, autophagy enhancing or inhibiting drugs were tested to evaluate their effects on H_2O_2- or MG-induced cell damage. We found that H_2O_2 (100 - 600 μM, n=4) dose-dependently decreased cell viability as determined by measuring live cell dehydrogenase activity. H_2O_2 (600 μM, n=4) decreased cell viability to 30 ± 5% of the control. Similarly, MG (400 - 1200 μM, n=5) decreased cell viability in a dose-dependent manner. MG (1200 μM, n=5) decreased cell viability to 30 ± 6% of the control. These results were further confirmed with the use of calcein staining, which contained calcein-AM for labeling live cells. By using Cyto ID autophagy detection dye, we found that H9c2 cells exhibited higher autophagy after incubation of H_2O_2 (100 - 600 μM, n=2) or MG (400 - 1200 μM, n=2), for 1 and 4 hours, and 1 and 24 hours, respectively. Intracellular ROS was also detected, and the data suggested that ROS levels increased as the dose of H_2O_2 and MG increased. Experimentation using autophagy regulatory drugs revealed that trehalose (500

μM - 100 mM, n=4), an autophagy enhancer, increased cell viability at 24 hours for cells exposed to H_2O_2 (600 μM), and increased cell viability at 1, 4, and 24 hours for cells exposed to MG (1200 μM) compared to the positive controls (600 μM H_2O_2 or 1200 μM MG). Trehalose (100 mM, n=4) increased cell viability by 78 ± 31% in MG-treated cells when compared to cells treated with MG (1200 μM) alone at one-hour post treatment. Trehalose also decreased intracellular ROS levels. Rapamycin (100 nM – 20 μM), another autophagy enhancer, reduced intracellular ROS levels in cells exposed to H_2O_2 and cells exposed to MG, but generally did not increase cell viability for cells exposed to 600 μM H_2O_2 or 1200 μM MG. 3-methyladenine (3-MA), an autophagy inhibitor, increased intracellular ROS levels. 3-MA (50 – 10,000 μM) provided no increase in cell viability compared to H_2O_2 (600 μM), but for MG at 1 and 4 hours, 3-MA did increase cell viability. Our data suggests that trehalose has potential to provide protection against H_2O_2 or MG-induced damage, while rapamycin does not. 3-MA seems to provide protection against MG, but not H_2O_2.

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INTRODUCTION

Cardiovascular disease (CVD) is the main cause of death for people of all races and ethnicities in the United States. About one in every four deaths in America can be attributed to CVD. Risk factors such as high cholesterol, high blood pressure, and smoking can lead to CVD and stroke; a little less than half of Americans have at least one of the three risk factors. Medical conditions, such as diabetes, can increase the risk of a person suffering from CVD. Certain lifestyle choices can also increase the chances of CVD occurring, such as an unhealthy diet, being overweight, abusing alcohol, etc. (Center for Disease Control, 2019). Under normal conditions, the heart will pump blood to the rest of the body so that nutrients and oxygen can reach organs for proper functioning. Many cardiovascular diseases impede this blood flow, therefore stopping the nutrients and oxygen from getting to their needed locations in the body. CVDs prevent the flow of blood by damaging and reshaping blood vessels; this can occur in the form of making the vessels harder and narrower therefore reducing blood flow (Cervantes et al, 2017). Oxidative stress, which serves as an early causative factor of CVDs, can damage cardiomyocytes, therefore decreasing heart function even more (Cervantes et al, 2017).

1.1 Oxidative Stress

Under normal conditions, reactive oxygen species (ROS) are formed endogenously as by-products of cellular metabolism mechanisms. ROS levels are low when cellular function is not disrupted. One way that ROS levels are maintained at a low level is by cellular antioxidant mechanisms. If ROS are overproduced, they can initiate a vicious cycle that facilitates the pathogenesis of various diseases, such as cancer,

diabetes, CVDs, and others. ROS threaten cells by reacting with DNA, proteins, and lipids and causing damage to them. Overproduction of ROS that exceeds the ability of the antioxidant mechanisms of the cell causes oxidative stress. When a cell is under oxidative stress, stress response mechanisms (e.g., antioxidant enzymes upregulation) are activated to help the cell adapt to the stress or resist it. Some of the activated stress response mechanisms are used to help the cell survive, while others induce cell death (Martindale and Holbrook, 2002; Navarro-Yepes et al, 2014).

ROS can contribute to the pathophysiology of cardiovascular disease. Damaged myocardiocytes can produce elevated concentrations of ROS within their mitochondria. Long-term ROS production in the mitochondria can cause mitochondrial DNA damage and mitochondrial functional decline (Tsutsui et al, 2011). This means that the myocardiocytes will no longer be receiving the necessary energy needed for normal function which can lead to cell death. High levels of ROS can also signal for apoptosis, a type of programmed cell death in myocardiocytes (Tsutsui et al, 2011). The more myocardiocytes die as a result of oxidative stress, the less the heart is able to function properly and pump blood to the rest of the body (Tsutsui et al, 2011).

1.1.1 Reactive Oxygen Species

ROS are small molecules that are highly reactive, since they can bind to cellular biomolecules disrupting normal function (Rezda-Dutordoir and Bates, 2016). The production of ROS is generally in equilibrium with antioxidant reactions keeping the ROS levels at an optimum level in the cell under physiological conditions. Moreover, low amounts of ROS are needed for normal cell functioning. ROS helps cell cycle

progression, cell proliferation, cell differentiation, and cell migration along with many other beneficial functions (Rezda-Dutordoir and Bates, 2016). ROS can activate cell signaling pathways and transcription factors, such as phosphoinositide 3-kinase (PI3K). PI3K is important in signaling for the activation of autophagy (Rezda-Dutordoir and Bates, 2016), which will be discussed later.

ROS can be formed by the cell both exogenously and endogenously. Exogenous sources of ROS production include ultraviolet light, ionizing radiation, and xenobiotics (Milkovic et al, 2019). Endogenous ROS is often in the form of superoxide, which is derived from in the mitochondrial electron transport chain (ETC). The main sites in the ETC of superoxide production are complexes I and III (Rezda-Dutordoir and Bates, 2016). In the ETC, about 2-3% of free electrons leak out and transfer to molecular oxygen forming superoxide radicals (Rezda-Dutordoir and Bates, 2016). Superoxide can also be formed by NADPH oxidases and xanthine oxidase located at the membrane (Rezda-Dutordoir and Bates, 2016). Superoxide is converted into H_2O_2 by the enzyme superoxide dismutase. High concentrations of H_2O_2 can form hydroxyl radicals ($OH^{\cdot}$) through the Fenton reaction (Rezda-Dutordoir and Bates, 2016). Hydroxyl radicals are quite dangerous since they are very reactive with biological macromolecules. To avoid the dangers that hydroxyl radicals can cause, H_2O_2 is converted to H_2O by catalase (Rezda-Dutordoir and Bates, 2016).

1.1.2 Hydrogen Peroxide-induced Oxidative Stress, Cell Damage, and Disease

As mentioned before, superoxide radicals form mostly in the mitochondria in the ETC. Superoxide radicals are often catalyzed by a nonenzymatic electron transfer reaction (Phaniendra et al, 2015). Superoxide radicals generally have low reactivity with

biomolecules. Their toxicity is more prominent through production of secondary free radicals (Phaniendra et al, 2015). For example, when two superoxide radicals react with one another in a dismutation reaction, one radical is oxidized to form oxygen, while the other superoxide radical is reduced to form H_2O_2 (Phaniendra et al, 2015). H_2O_2 can be much more damaging, because it can be converted into a hydroxyl radical when reacted with superoxide. Hydroxyl radicals are formed by the Fenton reaction, as previously mentioned. The Fenton reaction occurs when H_2O_2 reacts with metal ions, such as Fe^{2+} or Cu^{+} (Phaniendra et al, 2015). Fe^{2+} and Cu^{+} are often bound to different proteins, such as ferritin, which is an intracellular protein that stores iron, and ceruloplasmin, which is a copper-carrying protein found in the plasma (Phaniendra et al, 2015). When there is a high concentration of superoxide radicals, this stress will cause ferritin to release free Fe^{2+}, and the released Fe^{2+} will then participate in the Fenton reaction with H_2O_2 forming hydroxyl radicals (Phaniendra et al, 2015). Hydroxyl radicals cause more damage to the cell than any other ROS and react very strongly with inorganic and organic molecules. Hydroxyl radicals often react with DNA, proteins, lipids, and carbohydrates; when these structures in the cell are damaged, it can be detrimental to the cell (Phaniendra et al, 2015).

Oxidative stress caused by H_2O_2 can have a negative impact on cardiomyocyte DNA and ATP levels. Oxidative stress can cause the loss of purine and pyrimidine nucleotides in cardiomyocyte DNA; this can lead to ATP and phosphocreatine depletion (Janero et al, 1993). Phosphocreatine is important for transferring high energy phosphates to ADP. When phosphocreatine is depleted, ATP will not be able to form which will in turn provide less energy for cardiomyocytes eventually causing cardiomyocyte death

(Janero et al, 1993). A study was done to portray how H_2O_2 effects the energy levels in cardiomyocytes. The rate of nucleotide loss was dependent on the dosage of H_2O_2 (50 μM - 1 mM). At higher concentrations of H_2O_2, significant energy was lost in cardiomyocytes as a result of phosphate loss from nucleotide deletions. The only way to prevent ATP loss during H_2O_2-induced oxidative stress was dismutation of H_2O_2 (Janero et al, 1993). This is why H_2O_2 can be so detrimental to cardiomyocytes and the heart as a whole.

H_2O_2 increases under disease conditions, such as ischemia/reperfusion injury and diabetes (Slezak et al, 1995). A study was done to examine amounts of H_2O_2 present in myocardial cells of rats when the rat heart was under ischemia/reperfusion conditions. Within minutes of ischemia, the rat's heart lost its contractile ability, and the myocardial H_2O_2 levels increased 250% compared to control levels. Then, in the early stages of reperfusion, H_2O_2 concentrations were increased 600% compared to the control (Slezak et al, 1995). High blood sugar in diabetes also increases H_2O_2 concentrations (Ellis et al, 2000). A study was done comparing the amounts of plasma H_2O_2 levels in rats with diabetes and rats without diabetes. The rats with diabetes contained about twice as much H_2O_2 in their blood vessels than the rats that did not have diabetes (Ellis et al, 2000).

1.1.3 Methylglyoxal (MG)-induced Dicarbonyl Stress and Cell Damage

MG, a dicarbonyl compound, is a product of cellular metabolism. It is found in all cells. MG is produced via both enzymatic and non-enzymatic pathways (Allaman et al, 2015). MG can be made as a by-product in the pathways of protein synthesis and fatty acid metabolism, but glycolysis is the most significant endogenous source of MG production (Allaman et al, 2015). In the glycolysis pathway, MG is produced by the

fragmentation of glyceraldehyde-3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP) (Allaman et al, 2015). Normally, MG concentrations are very low in cells reaching up to no more than 300 μM. However, high MG levels can arise when the precursors in the production pathway are elevated (Allaman et al, 2015). For example, in diabetics, blood glucose concentrations are high producing high G3P and DHAP levels, therefore increasing MG concentrations above normal physiological concentrations (Allaman et al, 2015). Normally, MG is detoxified by glyoxalase I and II, which helps convert MG to D-lactate. D-lactate is then metabolized to pyruvate by D-lactate dehydrogenase (Bhowal et al, 2020).

When MG accumulate due to increased production or decreased detoxification, dicarbonyl stress occurs. Dicarbonyl stress is often known to occur with metabolic diseases, such as diabetes (Thornalley, 2008). MG is the most reactive dicarbonyl metabolite. MG is a glycating agent meaning that MG is involved in covalently attaching sugars to proteins and other cellular components, and as a result can induce cellular dysfunction by disrupting the normal function of these cellular components (Thornalley, 2008). Protein glycation consists of a complex series of reactions named collectively as the Maillard reaction (Thornalley, 2008). The Maillard reaction occurs in all tissues and bodily fluids. Later stages of glycation form what is known as advanced glycation end-products (AGEs), which can change protein structure, therefore changing its function (Thornalley, 2008). When MG reacts with proteins it forms AGE residues. When MG interacts with proteins the bond is irreversible (Thornalley, 2008). Specifically, MG reacts with the amino acid arginine to produce AGEs called hydroimidazolones

(Thornalley, 2008). AGEs have been shown to increase ROS levels as well as reduce catalase activity (Chen et al, 2018).

High levels of MG can be toxic to the cell because ROS production is increased. MG glycation often targets mitochondrial proteins. Glycation of these proteins causes an increase in ROS production (Desai et al, 2010). The ROS then react with other proteins, lipids, DNA, etc. therefore altering their structures and causing an alteration in function. High concentrations of ROS in the mitochondria will also alter the mitochondrial permeability, therefore disrupting proper mitochondrial function (Desai et al, 2010). MG also decreases the activity of superoxide dismutase, which is the first line of defense against oxidative stress and functions by converting superoxide to H_2O_2 (Desai et al, 2010).

1.1.4 Methylglyoxal Impact on Heart Cell Function

People who have diabetes are often at high risk for developing heart failure (Papadaki et al, 2018). MG is increased in diabetics, therefore increasing AGEs; AGEs are understood to be the pathophysiological link between the two diseases (Papadaki et al, 2018). A study compared MG modification levels in myofilaments of patients with diabetes and heart failure and in myofilaments of patients without diabetes and heart failure. The results showed that patients with diabetes and heart failure had an increase in MG modifications, therefore decreasing the function of myofilaments in heart cells (Papadaki et al, 2018).

Another study tested how MG affects a molecule called thioredoxin (Trx). Trx is cytoprotective and antiapoptotic molecule. It is important for cell survival. Inactivation of Trx has been known to be related to cardiovascular injury (Liu et al, 2010). Trx

inactivation can occur when AGEs glycate the molecule, since Trx is quite susceptible to glycation (Liu et al, 2010). An experiment was done to see if MG would cause Trx-glycative inactivation, therefore making cardiomyocytes more likely to undergo ischemia/reperfusion injury. When human Trx was incubated with MG, Trx glycation occurred rendering the molecule inactive, therefore making cardiomyocytes more prone to ischemia/reperfusion injury (Liu et al, 2010).

1.2 Autophagy

When cells are under stress, autophagy can be activated to cope with the stress. Autophagy is a term used to describe the process of a cell's damaged cytoplasmic components being degraded inside of a lysosome-fused vacuole. There are three types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy (Mizushima, 2007). The term "autophagy" is most often synonymous with macroautophagy (Mizushima, 2007). Generally speaking, the mechanism of autophagy begins with the formation of a phagophore, which then matures into a double-membraned autophagosome. The autophagosome and lysosome then fuse together forming what is known as an autolysosome. The autolysosome is where cellular components are broken down by the lysosome's degradative enzymes. The broken-down cellular components can then be recycled to establish homeostasis within the cell (Navarro-Yepes et al, 2014). This is a basic overview of the autophagy process; however, a more detailed description will be discussed in the following section.

Autophagy has been shown to have a vast range of purposes. It has physiological benefits as well as pathophysiological benefits. Some of the pros of autophagy include helping the cell adapt when the cell is not receiving adequate amounts of nutrients for

survival, clearing damaged intracellular proteins and organelles, tumor suppression, and more (Mizushima, 2007).

1.2.1 Autophagy Mechanism

The autophagy process begins with a membrane, which is known as a phagophore, and it is thought to be derived from the lipid bilayer of the endoplasmic reticulum or the Golgi apparatus (Glick et al, 2010). The phagophore expands and gathers intracellular materials, such as proteins, carbohydrates, lipids, etc. forming the autophagosome. The lysosome's proteases are then able to degrade the damaged macromolecules collected in the autophagosome that were gathered earlier in the process (Glick et al, 2010). The lysosome also has enzymes called permeases along with other transporters that function to export the degraded amino acids, monosaccharides, and other monomers back into the cytoplasm for the cell to reuse in order to build new macromolecules (Glick et al, 2010).

Autophagy is a complex process with many components, which can be seen in **Figure 1.1**. There are five significant stages: 1) nucleation (phagophore formation), 2) autophagy related protein 5-autophagy related protein 12 (Atg5-Atg12) conjugation, 3) light chain 3 (LC3) processing, 4) selection of cargo for degradation, and 5) autophagosome and lysosome fusion (Glick et al, 2010).

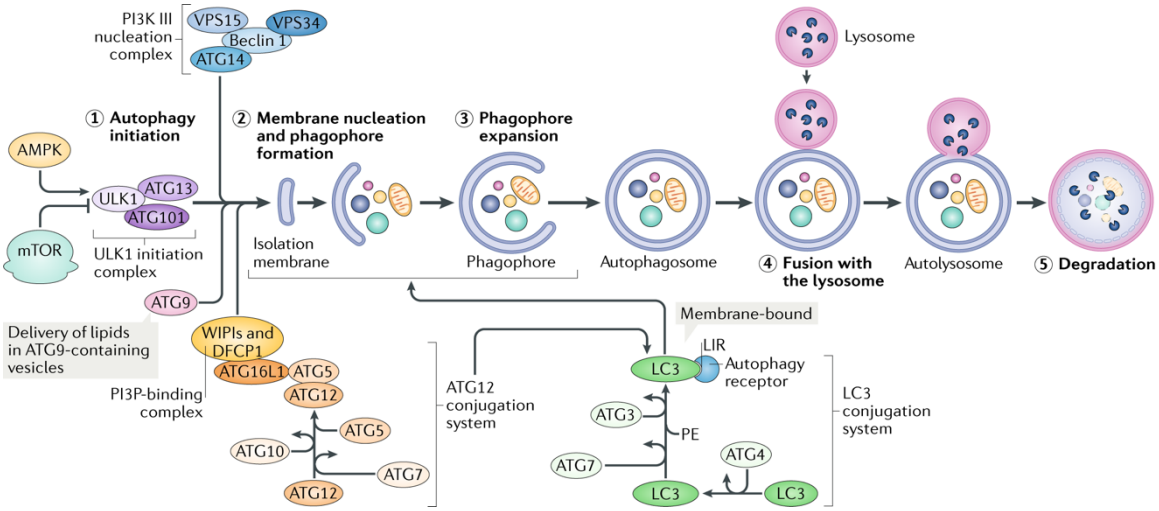

Figure 1.1 Autophagy Mechanism and the required proteins involved in initiating the formation of the phagophore, autophagosome, and autolysosome.
(Hansen et al, 2018).

Multiple signaling events take place in order for nucleation to occur. In yeast cells, Atg1 kinase works with Atg13 and Atg17 to form a phagophore. In mammalian cells, Ulk-1 is analogous with the yeast's Atg1 kinase (Glick et al, 2010). Ulk-1 functions to signal for autophagy to begin. Class III phosphatidylinositol-kinases (PI3K) are involved in autophagy and phagophore formation (Glick et al, 2010). One of the most notable class III PI3K proteins is vesicular protein sorting 34 (Vps34). When Vps34 is complexed with Beclin-1 and other regulatory proteins, it is involved in autophagy nucleation (Glick et al, 2010). Vps34 uses phosphatidylinositol as a substrate to form phosphatidylinositol triphosphate (PI3P). PI3P is required for phagophore elongation, and it also recruits other Atg proteins to the phagophore (Glick et al, 2010). Vps34 and Beclin-1 interaction increases the levels and activity of PI3P (Glick et al, 2010).

Atg5-Atg12 conjugation is important for elongation and closure of the autophagosome (Glick et al, 2010). It is a multistep process that begins with Atg7 binding to Atg12 in an ATP-dependent manner. Atg10 then is involved with mediating the

linkage between Atg12 to Atg5 (Glick et al, 2010). The conjugated Atg5-Atg12 complex then combines with Atg16L dimers to form an Atg5-Atg12-Atg16L complex that is involved with expanding the phagophore (Glick et al, 2010). Atg5-Atg12-Atg16L complex recruits LC3-II to assist in growing the phagophore and inducing the structure's curve (Glick et al, 2010). Once the phagophore completes its formation into the autophagosome, the Atg5-Atg12-Atg16L complex dissociates from the membrane (Glick et al, 2010).

LC3 is a microtubule-associated protein (Glick et al, 2010). LC3 is expressed in the cytosols of most cells. When autophagy is initiated, Atg4 proteolytically cleaves LC3 to form LC3-I (Glick et al, 2010). LC3-I is then activated in an ATP-dependent manner. Once LC3-I is activated, it is transferred to Atg3, then phosphatidylethanolamine (PE) is conjugated to glycine on LC3-I converting it to LC3-II (Glick et al, 2010). LC3-II is then recruited and integrated into the developing phagophore by the Atg5-Atg12-Atg16L complex (Glick et al, 2010). LC3-II is located on both the outer and inner surface of the autophagosome, and it functions to select the cargo in the cell's cytoplasm that needs to be degraded as well as hemifusion of the autophagosome's membranes (Glick et al, 2010). LC3 is a good protein to quantify to determine rate of autophagy since synthesis of LC3 increases during autophagy (Glick et al, 2010).

Autophagy had been thought to be a somewhat random process in the sense that the cargo engulfed in the autophagosome is indiscriminately gathered. Further research has shown that the membrane of the growing phagophore can actually interact with proteins, carbohydrates, lipids, and organelles to selectively uptake the cargo that requires

breakdown. LC3-II acts as a receptor to help determine which cytosolic materials should be taken in by the phagophore for degradation (Glick et al, 2010).

When the autophagosome formation is complete and the cargo deemed for degradation is carried within the structure, the next step in the process is fusion with the lysosome to form an autolysosome. Lysosomal proteases are delivered to the autophagosome and are then able to degrade the proteins, carbohydrates, lipids, and anything else stored inside the vesicle (Glick et al, 2010).

1.2.2 Autophagy, Oxidative Stress, and Cardiovascular Diseases

Autophagy actually has been proven to be beneficial in preventing CVDs, but it also has been proven to be dangerous. Autophagy has an adaptive phase and also a maladaptive phase (Mei et al, 2015). Adaptive autophagy in response to oxidative stress enhances myocardiocytes survival by getting rid of damaged organelles and recycling macromolecules (Mei et al, 2015). On the other hand, maladaptive autophagy is detrimental to myocardiocytes because there is an excess of self-digestion, and essential organelles and proteins get broken down. Maladaptive autophagy can also induce apoptosis (Mei et al, 2015). This can enhance the damage of CVDs.

Most studies have suggested that oxidative stress can induce autophagy. In contrast, antioxidant treatment can reduce autophagy related to oxidative stress. One study led by Danyong Li and Jingcheng Yu showed that H_2O_2 induced cell death by apoptosis in a dose-dependent (100 μM-1000 μM) and time-dependent (2 - 12 hours) manner in Mc3T3-E1 osteoblasts (Li et al, 2017). Moreover, cells exhibited a significant increase in autophagy after incubation with 500 μM and 1000 μM H_2O_2 for 8 hours. By

contrast, pretreating cells with catalase for one hour significantly reduced autophagy; however, cell viability after catalase treatment was not investigated in the study. The study also showed that application of an autophagy enhancer (e.g., rapamycin) reduced oxidative stress and cell apoptosis. Application of an autophagy inhibitor (e.g., 3-methyladeneine) significantly increased cell death when compared to H_2O_2 alone (Li et al, 2017).

On the other hand, some studies suggest that autophagy can facilitate cell apoptosis under oxidative stress. Ji Haye Ha and other researchers found that H_2O_2 dramatically induced H9c2 cell damage, and using propofol or autophagy inhibitors (e.g., wortmannin) can protect cells against H_2O_2-induced cell damage. More interestingly, H_2O_2 increased acidic autophagy vacuoles, whereas propofol largely reduced autophagy and its associated proteins (Ha et al, 2012). This study provides evidence of autophagy inducing more cell damage under oxidative stress.

MG has been known to increase the rate of autophagy just as H_2O_2 does. Specifically, the AGEs formed as a result of MG reacting with proteins are what cause the increase in the autophagy rate (Verma and Manna, 2016). AGEs will increase autophagy in various cell types as long as the cell has the receptor for AGE (RAGE), which cardiomyocytes do possess (Verma and Manna, 2016). A study was done to determine whether AGE would induce and increase autophagy in HepG2 cells, which, like cardiomyocytes, have RAGE (Verma and Manna, 2016). AGE (100 μg/mL) was added to the HepG2 cell line, and the cells were then stained with Monodansylcadaverine (MDC), which fluoresces in autophagy vacuoles. Results showed that MCD-stained HepG2 cells were consistently more fluorescent when exposed to AGE compared to the

control. This means that there were more autophagosomes formed as a result of AGEs. Using western blot analysis, autophagy proteins were analyzed when the cell line was exposed to AGE. AGE increased autophagy in a dose- and time-dependent manner as shown by an increase in the concentration of LC3 protein. These findings therefore support the claim that MG increases the rate of autophagy (Verma and Manna, 2016).

Some studies suggest that impaired autophagy may contribute to heart hypertrophy. Jia-Pu Wang accompanied by other researchers found that H9c2 cells treated with lower concentrations of H_2O_2 (10 - 50 μM) for 48 hours inhibited autophagy and accompanied cardiac myocyte hypertrophy. When 3-methyladenine (3-MA), an autophagy inhibitor, was also introduced to the H9c2 cells, even higher amounts of hypertrophy were noticed. In contrast, antioxidant administration reduced oxidative stress and cardiac hypertrophy. The results of the study showed that introducing antioxidants or restoring the myocardiocytes autophagy mechanisms could be useful in preventing myocardial hypertrophy (Wang et al, 2018).

In summary, there exists disparity regarding the role of autophagy under oxidative stress. Is autophagy helpful to cells, or is it harmful to the cells? It is also important to understand how to regulate the process of autophagy properly. With a better understanding of autophagy, the process could be used as a therapeutic method to treat or prevent CVD.

1.3 Autophagy Enhancers and Inhibitors

Introducing autophagy enhancers and inhibitors can help to understand the role of autophagy in human diseases. Adding autophagy enhancers and inhibitors to cells under oxidative stress can further our understanding of the mechanism behind autophagy and

help determine if the process will actually decrease oxidative stress in the cells or not. There are various autophagy enhancing and inhibiting drugs that target various proteins or receptors in the autophagy process. Enhancers used in this project include rapamycin and trehalose. The inhibitor used in this project is 3-MA.

1.3.1 Rapamycin

Rapamycin is an autophagy enhancing drug that targets the autophagy inhibitor protein mammalian target of rapamycin (mTOR) (Yang et al, 2013). Rapamycin binds fk506-binding protein 12kDa (FKBP12) forming a complex that inhibits mTOR activity (Yang et al, 2013). mTOR is one of the main regulatory pathways of autophagy. mTOR acts as an inhibitor of the autophagy process (Yang et al, 2013). Introduction of rapamycin works to inhibit mTOR and enhance Ulk-1's kinase activity, therefore signaling for autophagy to proceed (Yang et al, 2013).

1.3.2 Trehalose

Trehalose is a disaccharide sugar found in organisms, such as bacteria, plants, insects, fungi, and other invertebrates. It is not naturally found in mammals (Hosseinpour-Moghaddam et al, 2018). Trehalose prevents protein denaturation in these organisms, so it helps protect against various stress conditions (Hosseinpour-Moghaddam et al, 2018). Trehalose, when introduced into mammals, works as an enhancer of autophagy. Trehalose works by inhibiting transport of glucose and fructose through glucose (GLUT) transporters (Mardones, Rubinsztein, and Hetz, 2016). This forces the cell into a starvation-like state that will then signal the autophagy process to take place. Autophagy is stimulated through AMP-activated protein kinase (AMPK) and activation

of Ulk-1 (Mardones, Rubinsztein, and Hetz, 2016). This is an mTOR-independent mechanism (Mardones, Rubinsztein, and Hetz, 2016).

1.3.3 3-Methyladenine

3-MA is an autophagy inhibitor. 3-MA inhibits autophagy by inhibiting the class III PI3K complex. Inhibiting class III PI3K will then block PI3P from forming (Wu et al, 2010). Other Atg proteins would normally recruit PI3K to signal for the initiation of autophagy, but since PI3K is blocked in this case, initiation does not occur, therefore autophagy ceases (Wu et al, 2010).

1.3.4 Previous Study of Autophagy during I/R in the lab

One of my peers, Aloysius Ibe, did a research study on a similar topic. In fact, this particular project is an extension of his project. His project's focus was on determining if autophagy benefits the heart when an ischemic/reperfusion insult takes place. He used the same autophagy regulatory drugs as I did for this project: rapamycin, trehalose, and 3-MA. Aloysius's project tested the effects of the autophagy enhancers (rapamycin, trehalose) and the autophagy inhibitor (3-MA) on heart function and infarct size after ischemia/reperfusion. The results from his study suggested that autophagy enhancers reduced Ischemia/reperfusion injury.

Similarly to Aloysius's project, our project uses the same autophagy regulatory drugs (rapamycin, trehalose, and 3-MA). Instead of focusing on I/R injury and infarct sizes in particular, our project focused on reducing the levels of ROS in myocardiocytes that can be raised by diseases like I/R injury, CVDs, and diabetes. In our study, oxidative stress was induced by increasing concentrations of H_2O_2 or MG. We used the autophagy

regulatory drugs to determine whether enhancement of autophagy or downregulation of autophagy could appease the levels of ROS, therefore decreasing oxidative stress in myocardiocytes and reducing cardiac damage.

1.4 Hypothesis

We hypothesize that H_2O_2 and MG will decrease cell viability in a dose-dependent and time-dependent manner and will also increase ROS and oxidative stress in cardiomyoblasts. We also hypothesize that enhancement of autophagy would help decrease cardiac myocyte oxidative-stress-induced or dicarbonyl-stress-induced cell damage.

METHODS

2.1 H9c2 Myoblast Cell Culture

H9c2 cells were used for experimentation. In order to perform various experiments, the H9c2 cells required proper maintenance. The H9c2 cells needed to have a confluence of about 70-80%, otherwise the cell population would die out. To maintain the cell line, the cell culture required subculturing. Subculturing entailed discarding old medium containing the cells' metabolic wastes, then washing the cell line with phosphate buffered saline (PBS). Once the cells were washed with PBS, 2.5% trypsin (Sigma Aldrich, St Louis, MO, USA) was added to the cells. Trypsin was necessary to allow the cells to free themselves from the bottom of the flask. The cell culture was then added to a collecting tube and centrifuged (1000rpm for 7 minutes). The supernatant was discarded after centrifuging, then new medium was added to the cells to achieve the desired concentration of 2×10^5 cells/mL. Thereafter, 100 µL of cell suspension (2×10^4 cells) were added to each well in a 96-well plate. After a 24-hour incubation period in the 96-well plate, the cells were able to attach themselves to the bottom of the well and were ready for experimentation.

2.2 Dose response: H_2O_2 and MG

H_2O_2 (100 µM - 600 µM) was added to the cells to determine the effect of H_2O_2 on H9c2 cell viability after the 1, 4, or 24-hr incubation period. Similarly, MG (400 µM - 1200 µM) was added to the cells to determine the effect of MG on H9c2 cell viability after a 1, 4, or 24-hr incubation period.

2.3 H_2O_2 (600 μM) + Rapamycin & MG (1200 μM) +Rap

Medium/solvent alone, H_2O_2 (600 μM), and H_2O_2 (600 μM) combined with rapamycin (25 nM - 20 μM) were added to cells to determine if enhancement of autophagy by rapamycin could protect cells against H_2O_2-induced cell damage after a 1, 4, or 24-hr incubation period.

Similarly, medium/solvent alone, MG (1200 μM), and MG (1200 μM) combined with rapamycin (25 nM - 20 μM) were added to cells to determine if enhancement of autophagy by rapamycin could protect cells against MG-induced cell damage after a 1, 4, or 24-hr incubation period.

2.4 H_2O_2 (600 μM) + Trehalose & MG (1200 μM) + Trehalose

Similar to the previous experiment, medium/solvent alone, H_2O_2 (600 μM), and H_2O_2 (600 μM) combined with trehalose (100 μM - 100 mM) were added to cells to determine if enhancement of autophagy by trehalose could protect cells against H_2O_2-induced cell damage after a 1, 4, or 24-hr incubation period.

Medium/solvent alone, MG (1200 μM), and MG (1200 μM) combined with trehalose (100 μM - 100 mM) were added to cells to determine if enhancement of autophagy by trehalose could protect cells against MG-induced cell damage after a 1, 4, or 24-hr incubation period.

2.5 H_2O_2 (600 μM) + 3-MA & MG (1200 μM) + 3-MA

This experiment followed a similar format as the previous experiments; however, instead of using an autophagy enhancing drug, the autophagy inhibitor 3-MA was used.

Increasing concentrations of 3-MA (10 μM - 10 mM) with H_2O_2 (600 μM) or MG (1200 μM) was used to determine if inhibition of autophagy by 3-MA could protect cells against MG or H_2O_2-induced cell damage after a 1, 4, or 24-hr incubation period.

2.6 Cell Counting Kit (CCK) Assay

CCK assay was used to determine the number of viable cells based on dehydrogenase activity in live cells. CCK uses tetrazolium salt, which is converted to formazan dye by intracellular dehydrogenase. Formazan dye is orange in color. The greater the amount of formazan dye, the higher the number of living cells there are and the darker the orange stain appears. After 1, 4, and 24-hr incubation, the old medium containing H_2O_2, MG, or H_2O_2/MG with enhancing or inhibiting drug was removed, and cells were washed with PBS twice. Then, 100 μL of fresh medium was added, and 10 μL of CCK assay buffer was put into each well. Absorbance was measured by an ELISA plate reader at 450 nm wavelength after 4-hr incubation with the CCK assay buffer. The cell viability was expressed as a ratio by calculating absorbance relative to medium/solvent control or 600 μM H_2O_2/1200 μM MG control.

2.7 Calcein-AM Staining

Calcein-AM staining was used to stain live cells. Calcein-AM is a cell membrane permeable compound. Calcein generated by esterase activity in living cells, emits strong green fluorescence, so viable cells could be monitored as green fluorescent-stained cells under a fluorescent microscope. After drug treatment for 1, 4, or 24-hrs, medium was removed, then 100 μL of PBS containing Calcein-MA (.001 M) was added to cells. After

a 30-minute incubation period, florescence was measured at 485 nm/535 nm. The ratio to the medium/solvent control or H_2O_2/MG control was calculated.

2.8 Intracellular ROS detection by 2',7'-dicholorofluorescein diacetate (DCFDA)

DCFDA was used to detect intracellular ROS levels. DCFDA is a non-fluorescent, cell permeable reagent. Once DCFDA enters the cell it is deacetylated by cellular esterases, then it is oxidized by intracellular ROS forming 2',7'-dichlorofluorescein (DCF). DCF is a fluorescent compound that can be detected using fluorescence spectroscopy.

Before exposure to oxidative stress via H_2O_2 or MG, the H9c2 cells were washed twice with PBS and incubated for 45 minutes with 100 μL DCFDA assay (.025 M) in working buffer (Hank's Balances Salt Solution and Fetal Bovine Serum). After about 45 minutes, the cells were washed again with PBS twice to remove the DCFDA from the incubating solution. The fluorescent DCF were then observed under the Fluoroskan Ascent CF scanner to get a base line reading before any treatment. After the reading, different concentrations of H_2O_2 and MG were added to the cell culture along with the autophagy enhancers and inhibitor described previously, which were prepared during the 45-minute incubation period. After treatment, the fluorescent DCF were measured under the Fluoroskan Ascent CF scanner at 10 minutes post treatment, 30 minutes post treatment, 1hr post treatment, 2hr post treatment, 6hr post treatment, and 24hrs post treatment.

2.9 Cyto-ID Autophagy Detection Kit

Cyto-ID (Enzo Life Sciences) was used to detect autophagy in live cells by fluorescence microscopy and Fluoroskan. Cyto-ID was a fairly quick method supplying quantitative data for monitoring autophagy activity at a cellular level. Cyto-ID involves a green fluorescent detection reagent that is excitable at 488 nm. This reagent fluoresces brightly in autophagy vesicles.

The steps involved for Cyto-ID for a live cell analysis by fluorescence microplate reader are as follows: the H9c2 cells were seeded in a 96-well plate the day prior to the experiment. This allows the cells to attach overnight. The following day, the cells were treated with H_2O_2, MG, and the respective autophagy modulating drugs. After 1, 4, or 24 hours of exposure to the treatment, the medium was removed and 100 μL 1X Assay Buffer (9 mL deionized water and 1 mL 10X Assay Buffer from kit) was added to each well. The 1X Assay Buffer was then immediately removed and 100 μL of dual color detection solution was added to each well. The dual color detection solution was made by combining 9 mL of 1X Assay Buffer with 18 μL of CYTO-ID Green detection reagent and 9 μL of Hoechst 33342 Nuclear Stain. Once the dual color detection solution was added to each well, the plate was wrapped in aluminum foil to protect it from the light and then incubated at 37 degrees Celsius for 30 minutes. After 30 minutes, the cells were washed twice with 200 μL of 1X Assay Buffer in order to remove any extra dye in the wells. Then 100 μL of 1X Assay Buffer was added to each well. The plate was then ready to be read in a fluorescence microplate reader. CYTO-ID Green detection reagent was read at 488 nm excitation, 527 nm emission. Hoechst 33342 Nuclear Stain was read at 355 nm excitation, 490 nm emission.

2.10 Data Analysis and Statistics

The experiments were performed in duplicate and repeated four times, then the mean of the duplicate treatments was recorded. Data was expressed as means ± S.E.M. The data related to intracellular ROS and autophagy were not analyzed by statistics due to very small sample size as they were done in duplicates and repeated only two to three times. By contrast, the data generated by CCK and calcein assay were analyzed using two-way ANOVA followed by Tukey's post hoc test, and $p < 0.05$ suggested significant findings.

RESULTS

3.1 Cell Damage caused by H_2O_2 or MG

3.1.1 Hydrogen Peroxide Decreases H9c2 Cell Viability

The effects of H_2O_2 (100 - 600 µM, n=4-5) on H9c2 cell viability after CCK assay at 1, 4, and 24 hours are shown in **Figure 3.1**. At a concentration of 100 µM, H9c2 cell viability remained the same as the control or slightly increased. From 300 - 600 µM H_2O_2 cell viability decreased in a dose-dependent manner, but not in a time-dependent manner ($p < 0.05$ vs negative control). H_2O_2 600 µM significantly reduced cell viability to 28% ± 3%, 30% ± 5%, and 17% ± 1% relative to the nontreated control cells (100% cell viability) at 1, 4, and 24 hours, respectively ($p < 0.05$ vs negative control). H_2O_2 600 µM was chosen to further test effects of autophagy enhancers or inhibitors on H_2O_2-induced cell damage.

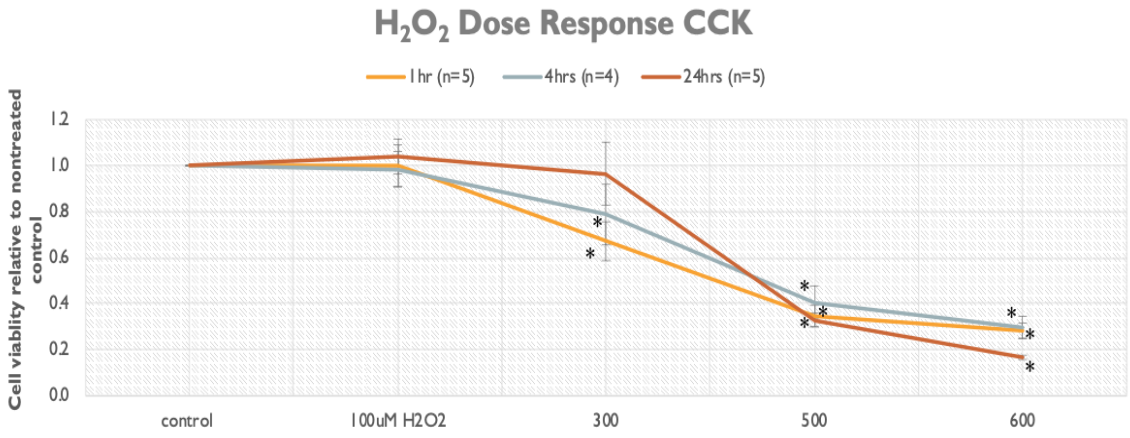

Figure 3.1 H_2O_2 dose response on cell viability by CCK assay. H_2O_2 reduced cell viability in a dose-dependent, but not a time-dependent manner (*$p < 0.05$ compared to negative control). Cell

viability was measured at 1, 4, and 24 hours after adding increasing doses of H_2O_2 to the cardiomyoblasts.

The effects of H_2O_2 (100 - 600 µM, n=4-6) on H9c2 cell viability by calcein stain at 1, 4, and 24 hours are shown in **Figure 3.2**. Calcein stain was used as a separate test to confirm the CCK cell viability being that both assays determine cell viability via different intracellular enzymes. Calcein stain data showed that 100 - 600 µM H_2O_2 decreased cell viability compared to the nontreated (negative) control in a dose-dependent but not time-dependent manner ($p < 0.05$ vs negative control). H_2O_2 600 µM significantly reduced cell viability to 36% ± 5%, 27% ± 1%, and 21% ± 2% relative to the nontreated control (100% cell viability) at 1, 4, and 24 hours, respectively ($p < 0.05$ vs negative control).

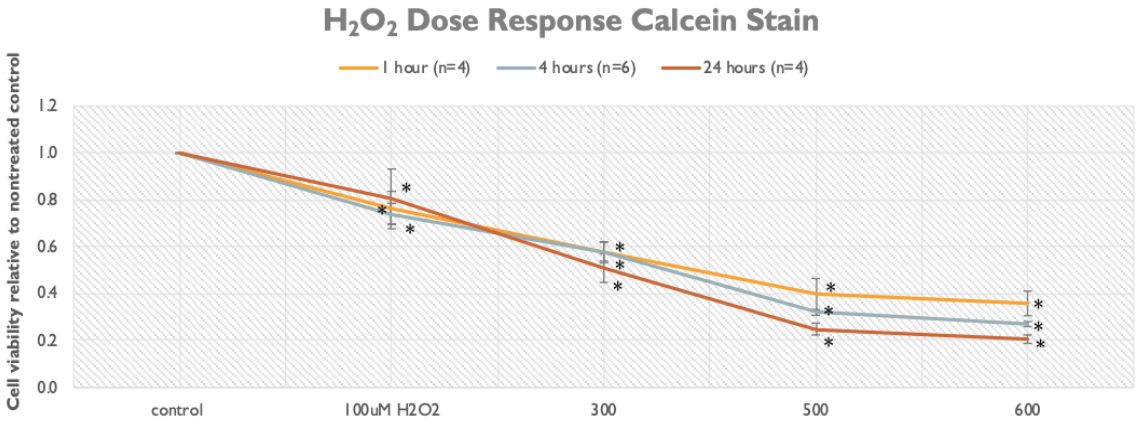

Figure 3.2 H_2O_2 dose response on cell viability by calcein stain assay. H_2O_2 reduced cell viability in a dose-dependent, but not a time-dependent manner (*$p < 0.05$ compared to negative control). Cell viability was measured at 1, 4, and 24 hours after adding increasing doses of H_2O_2 to the cardiomyoblasts.

3.1.2 Methylglyoxal Decreases H9c2 Cell Viability

The effects of methylglyoxal (400 µM - 1200 µM, n=3-5) on H9c2 cell viability via CCK at 1, 4, and 24 hours are shown in **Figure 3.3**. At a concentration of 400 µM,

H9c2 cell viability remained the same as the control or slightly increased, so there was no statistical difference at 400 µM MG when compared to the negative control. From 800 - 1200 µM, MG significantly decreased cell viability in a dose-dependent manner but not a time-dependent manner compared to the negative control ($p < 0.05$ vs negative control). MG 1200 µM significantly reduced cell viability to 33% ± 3%, 36% ± 7%, and 30% ± 6% relative to the nontreated control (100% cell viability) at 1, 4, and 24 hours, respectively ($p < 0.05$ vs negative control). MG 1200 µM was chosen to further test effects of autophagy enhancers or inhibitors on MG-induced cell damage.

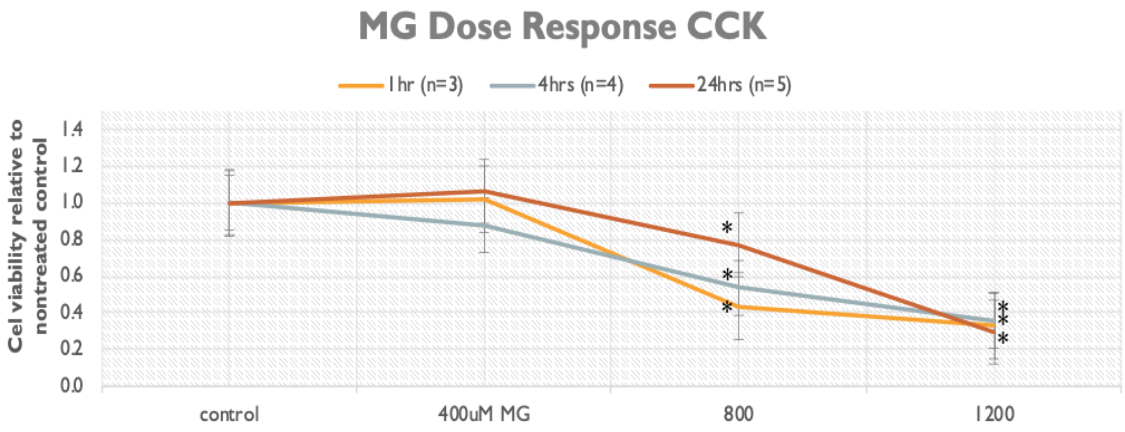

Figure 3.3 MG dose response on cell viability by CCK assay. MG reduced cell viability in a dose-dependent, but not a time-dependent manner (*$p < 0.05$ compared to negative control). Cell viability was measured at 1, 4, and 24 hours after adding increasing doses of MG to the cardiomyoblasts.

The effects of methylglyoxal (400 µM - 1200 µM, n=4-6) on H9c2 cell viability via calcein stain at 1, 4, and 24 hours are shown in **Figure 3.4**. MG concentrations 400 µM – 1200 µM decreased cell viability significantly compared to the negative control in a dose dependent manner but not a time dependent manner ($p < 0.05$ vs negative control).

MG 1200 µM significantly reduced cell viability to 33% ± 5%, 19% ± 1%, and 17% ± 1% relative to the nontreated control (100% cell viability) at 1, 4, and 24 hours, respectively ($p < 0.05$ vs negative control).

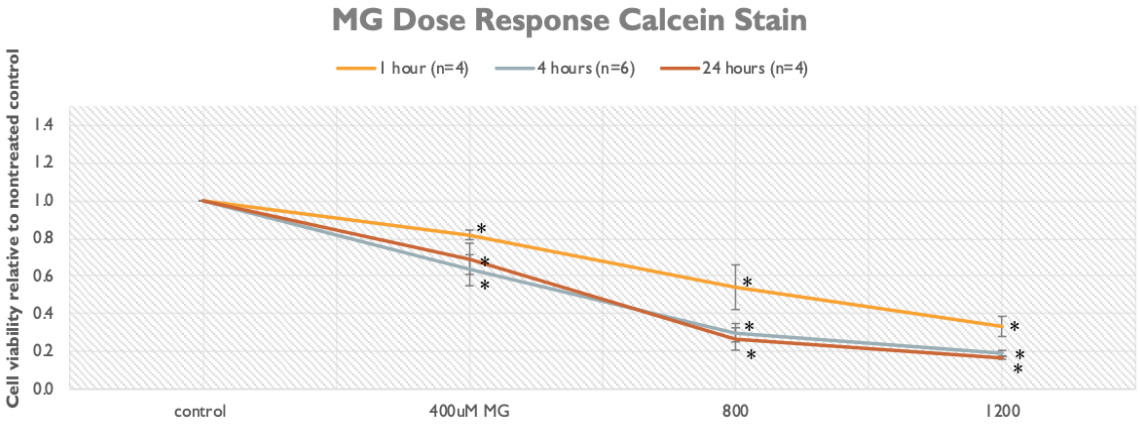

Figure 3.4 MG dose response on cell viability by calcein stain assay. MG reduced cell viability in a dose-dependent, but not a time-dependent manner (*$p < 0.05$ compared to negative control). Cell viability was measured at 1, 4, and 24 hours after adding increasing doses of MG to the cardiomyoblasts.

3.1.3 Hydrogen Peroxide Increases Intracellular ROS in H9c2 Cells in a Dose- and Time-Dependent Manner

H_2O_2 (100 µM - 600 µM, n=2-3) increased intracellular ROS concentrations in H9c2 cells in a dose-dependent manner as shown in the DCFDA data in **Figure 3.5**. As portrayed in the graph, as the concentration of H_2O_2 increased, the level of ROS also increased. It can also be seen that as time increased, the concentration of ROS increased, especially for higher doses of H_2O_2, such as 500 – 600 µM. At 30 minutes, 500 and 600 µM H_2O_2 increased intracellular ROS levels 8.14 ± 1.58-fold and 17.69 ± 4.52-fold, respectively, when compared to the negative control. Moreover, at 24 hours 600 µM H_2O_2 increased intracellular ROS levels 32.54 ± 7.47 times that of the negative control.

Because the experimentation was only done 2-3 times, statistical analysis was not performed.

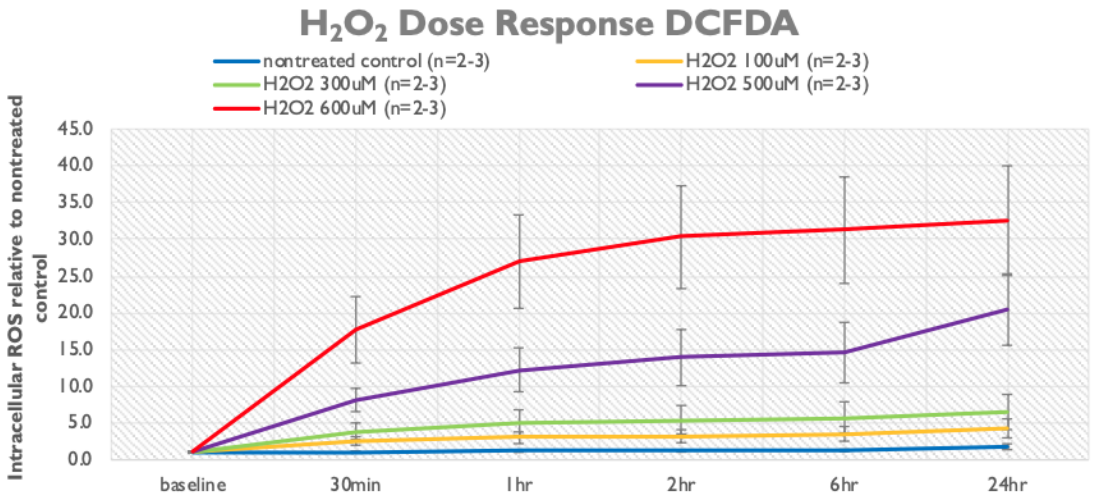

Figure 3.5 H_2O_2 dose response on intracellular ROS by DCFDA assay. H_2O_2 increased intracellular ROS levels in a dose-dependent and time-dependent manner. Cardiomyoblast intracellular ROS was measured from baseline – 24 hours after adding increasing doses of H_2O_2.

3.1.4 Methylglyoxal Increases Intracellular ROS in H9c2 Cells in a Dose and Time-Dependent Manner

MG (400 µM - 1200 µM, n=2-3) increased intracellular ROS concentrations in H9c2 cells in a dose- and time-dependent (0 minutes-24 hours) manner as shown in the DCFDA data in **Figure 3.6**. We saw that 400 µM and 800 µM actually showed very similar results in terms of increasing intracellular ROS. At 6 hours, 400, 800, and 1200 µM MG increased intracellular ROS levels 2.33 ± 0.31, 2.17 ± 0.23-fold, and 3.70 ± 0.91-fold respectively, when compared to the negative control. At 24 hours, 400, 800, and 1200 µM MG increased intracellular ROS levels 4.23 ± 0.72, 4.54 ± 0.62-fold, and 8.88 ± 2.58-fold, respectively, compared to the negative control. Because the

experimentation was only done 2-3 times, statistical analysis did not show any significant effects.

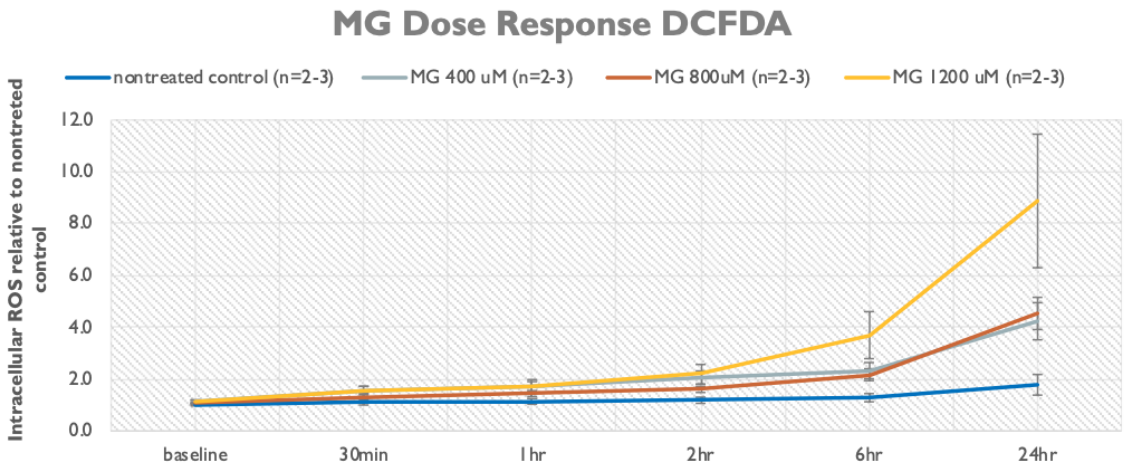

Figure 3.6 MG dose response on intracellular ROS by DCFDA assay. MG increased intracellular ROS levels in a dose-dependent and time-dependent manner. Cardiomyoblast intracellular ROS was measured from baseline – 24 hours after adding increasing doses of MG.

3.2 Oxidative Stress and Autophagy Regulatory Drugs

3.2.1 Hydrogen Peroxide and Trehalose

The effects of trehalose (500 µM - 100 mM, n=3-6) co-treated with H_2O_2 (600 µM) on H9c2 cell viability are shown in **Figure 3.7**. Trehalose exhibited protection against H_2O_2 in a dose- and time-dependent manner. Only the higher concentrations of trehalose (50 – 100 mM) showed slightly increased cell viability at 1 and 4 hours. However, at 24 hours, 5 mM - 100 mM trehalose significantly increased cell viability significantly compared to the positive control (600 µM H_2O_2) ($p < 0.05$ vs positive control). Meanwhile, higher concentrations of trehalose showed better protection. Trehalose (50 mM) at 24 hours showed maximal increased cell viability (2.01 ± 0.19

times that of the positive control; $p < 0.05$ vs positive control). The higher the y-axis value shown in the graph, the greater the number of viable cells in a well.

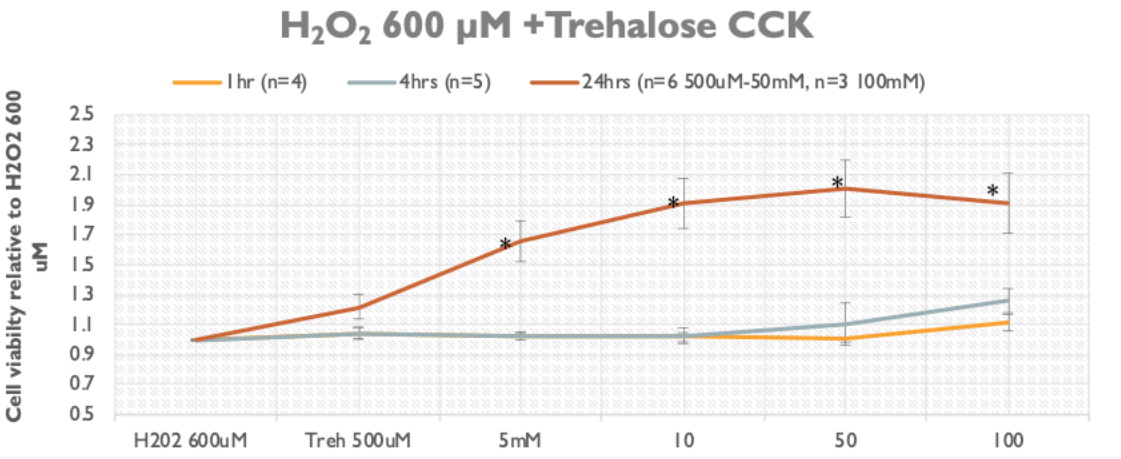

Figure 3.7 Trehalose cotreatment with 600 µM H_2O_2 effect on cell viability by CCK assay. Trehalose significantly increased cell viability only at 24 hours when compared to H_2O_2. (*$p < 0.05$ compared to H_2O_2 (600 µM) alone). Cell viability was measured at 1, 4, and 24 hours after adding 600 µM H_2O_2 with increasing doses of trehalose.

3.2.2 Methylglyoxal and Trehalose

The effects of trehalose (500 µM - 100 mM, n=4) co-treated with MG (1200 µM) on H9c2 cell viability are shown **Figure 3.8**. Trehalose (5 mM - 100 mM) exhibited significant protection against MG-induced cell damage in a dose-dependent manner at 1, 4, and 24 hours ($p < 0.05$ vs positive control). The same dose of trehalose showed similar cell viability at 1 and 4 hours. At 4 hours, trehalose (100 mM) increased cell viability to 1.96 ± 0.11 times that of the positive control ($p < 0.05$ vs positive control). At 24 hours, trehalose (5 mM) showed better fold improvement in cell viability (2.08 ± 0.47) than the higher concentration (100 mM: 1.71 ± 0.25).

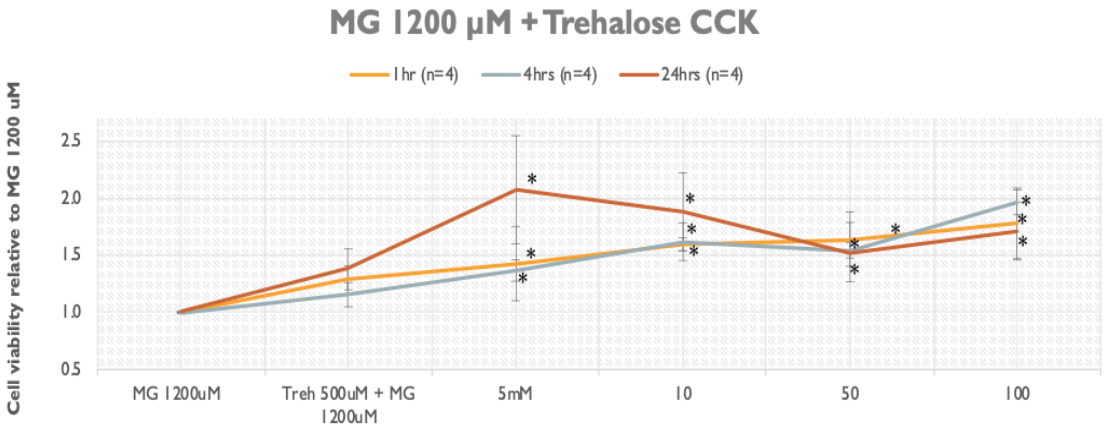

Figure 3.8 Trehalose cotreatment with 1200 µM MG effect on cell viability by CCK assay. Trehalose significantly increased cell viability at 1, 4, and 24 hours when compared to MG. (*p < 0.05 compared to MG (1200 µM) alone). Cell viability was measured at 1, 4, and 24 hours after adding 1200 µM MG with increasing doses of trehalose.

3.2.3 Hydrogen Peroxide and Rapamycin

The effects of rapamycin (100 nM - 20 µM, n=3-5) co-treated with H_2O_2 (600 µM) on H9c2 cells viability are shown in **Figure 3.9**. All doses of rapamycin did not show any significant improvement in cell viability compared to the positive control at 1, 4, and 24 hours.

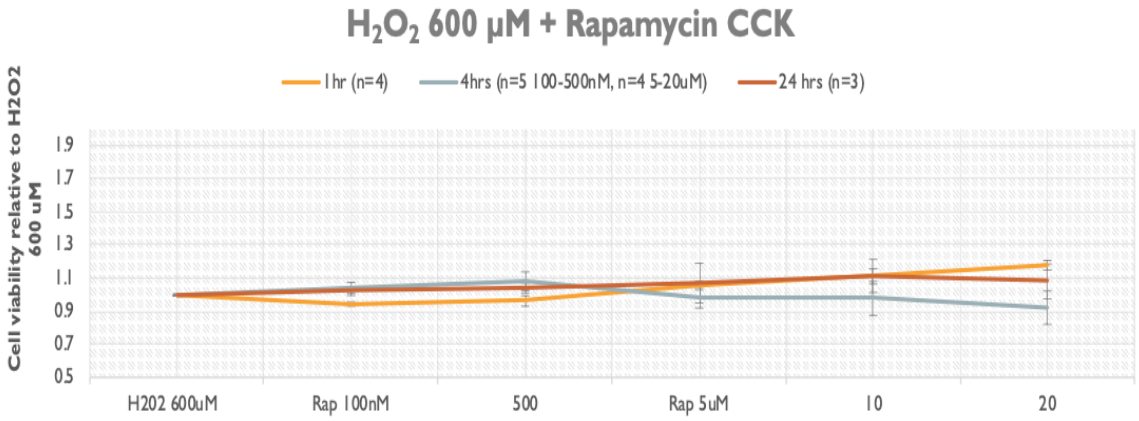

Figure 3.9 Rapamycin cotreatment with 600 µM H_2O_2 effect on cell viability by CCK assay. Rapamycin did not show any significant increase in cell viability when compared to H_2O_2. Cell

viability was measured at 1, 4, and 24 hours after adding 600 μM H_2O_2 with increasing doses of rapamycin.

3.2.4 Methylglyoxal and Rapamycin

The effects of rapamycin (100 nM - 20 μM, n=4) co-treated with MG (1200 μM) on H9c2 cells viability are shown in **Figure 3.10**. Rapamycin only showed slight improvement in cell viability at 4 hours but not 1 or 24 hours. Moreover, the improvement was not dose dependent. 100 nM rapamycin was the only dosage that significantly increased cell viability 1.48 ± 0.20-fold compared to the positive control ($p < 0.05$ vs positive control).

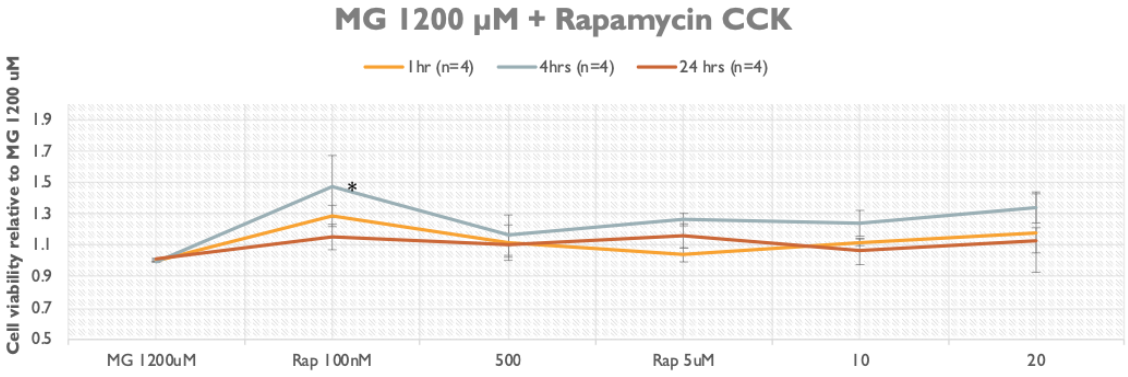

Figure 3.10 Rapamycin cotreatment with 1200 μM MG effect on cell viability by CCK assay. Rapamycin did not show significant increase in cell viability at the most doses when compared to H_2O_2 (*$p < 0.05$ compared to MG (1200 μM) alone). Cell viability was measured at 1, 4, and 24 hours after adding 1200 μM MG with increasing doses of rapamycin.

3.2.5 Hydrogen Peroxide and 3-Methyladenine

The effects of 3-methyladenine (50 μM - 10,000 μM, n=3-4) co-treated with H_2O_2 (600 μM) on H9c2 cells viability are shown in **Figure 3.11**. Compared to the positive

control (600 µM H_2O_2), there were no significant increases in cell viability at any dose of 3-MA at 1, 4, or 24 hours.

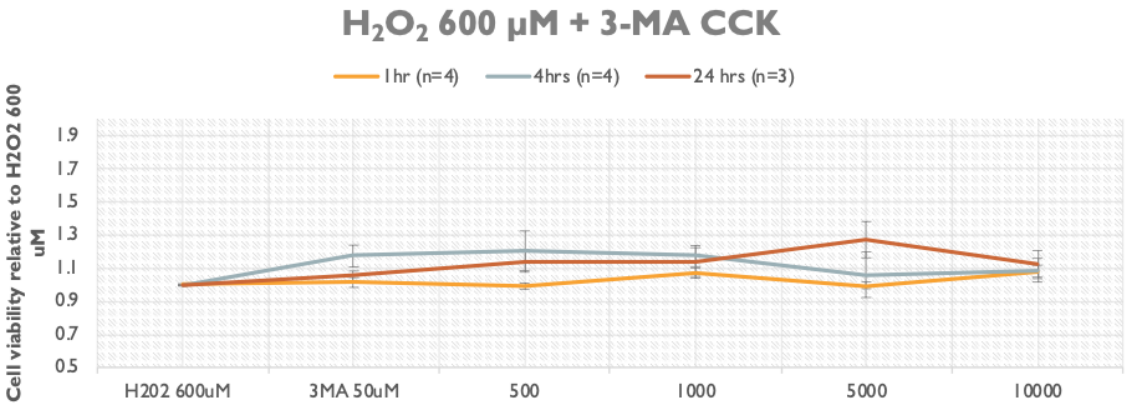

Figure 3.11 3-MA cotreatment with 600 µM H_2O_2 effect on cell viability by CCK assay. 3-MA did not show any significant increase in cell viability when compared to H_2O_2. Cell viability was measured at 1, 4, and 24 hours after adding 600 µM H_2O_2 with increasing doses of 3-MA.

3.2.6 Methylglyoxal and 3-methyladenine

The effects of 3-methyladenine (50 µM - 10,000 µM, n=3-4) co-treated with MG (1200 µM) on H9c2 cells viability are shown in **Figure 3.12**. 3-MA showed improved cell viability at 1 and 4 hours, but not 24 hours. Moreover, the protection was dose independent. At a concentration of 10,000 µM 3-MA, cell viability was increased 1.43 ± 0.26 and 1.43 ± 0.09 times that of the positive control at 1 and 4 hours, respectively, ($p < 0.05$ vs positive control).

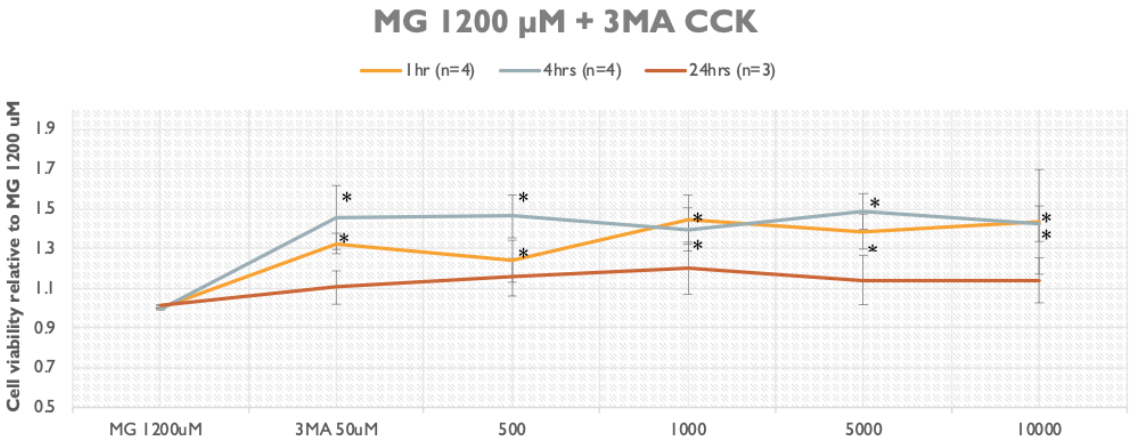

Figure 3.12 3-MA cotreatment with 1200 µM MG effect on cell viability by CCK assay. 3-MA significantly increased cell viability at 1 and 4 hours but not 24 hours when compared to MG. (*p < 0.05 compared to MG (1200 µM) alone). Cell viability was measured at 1, 4, and 24 hours after adding 1200 µM MG with increasing doses of 3-MA.

3.3 Reactive Oxygen Species and Autophagy Regulatory Drugs

3.3.1 Reactive Oxygen Species of H9c2 Cells exposed to H_2O_2 and Trehalose

The effects of trehalose (500 µM and 100 mM, n=2-3) co-treated with H_2O_2 (600 µM) on ROS levels of H9c2 cells are shown in **Figure 3.13**. H_2O_2 600 µM started to increase intracellular ROS levels at 30 min and continued throughout 24 hours when compared to nontreated control. By contrast, trehalose (500 µM and 100 mM) showed a trend to reduce H_2O_2-induced increase in ROS from 30 min to 24 hours. At 24 hours, H_2O_2 (600 µM) increased intracellular ROS levels 32.54 ± 7.47-fold when compared nontreated control. The higher the y-axis value in the DCFDA graph, the greater the amount of intracellular ROS. Trehalose (500 µM and 50 mM) reduced intracellular ROS levels to 21.75 ± 6.99 and 25.71 ± 7.74, respectively, when compared to 600 µM H_2O_2 alone. However, because of a small sample size, no statistics was able to be completed.

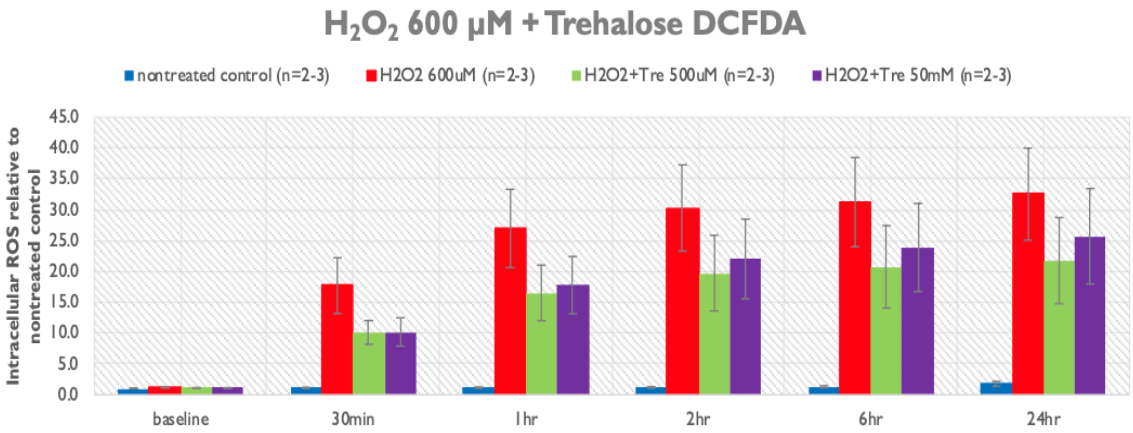

Figure 3.13 Trehalose cotreatment with 600 µM H_2O_2 effect on intracellular ROS by DCFDA assay. Trehalose showed a trend to reduce intracellular ROS levels when compared to H_2O_2. Cardiomyoblast intracellular ROS was measured from baseline – 24 hours after adding 600 µM H_2O_2 with increasing concentrations of trehalose.

3.3.2 Reactive Oxygen Species of H9c2 Cells exposed to MG and Trehalose

The effects of trehalose (500 µM - 100 mM, n=2-3) co-treated with MG (1200 µM) on ROS levels in H9c2 cells are shown in **Figure 3.14**. MG 1200 µM started to increase intracellular ROS levels at 2 hour and continued to raise it through 24 hours when compared to nontreated control. By contrast, the lower dose of trehalose (500 µM) but not the higher dose of trehalose (100 mM) showed a trend to reduce MG-induced increase in ROS from 2 hour to 24 hours. At 24 hours, MG (1200 µM) increased intracellular ROS levels 8.88 ± 2.58 times that of the nontreated control. By contrast, trehalose (500 µM) increased intracellular ROS levels only 3.75 ± 1.15 times that of the nontreated control. Therefore, trehalose (500 µM) decreases intracellular ROS compared to 1200 µM MG alone at 24 hours.

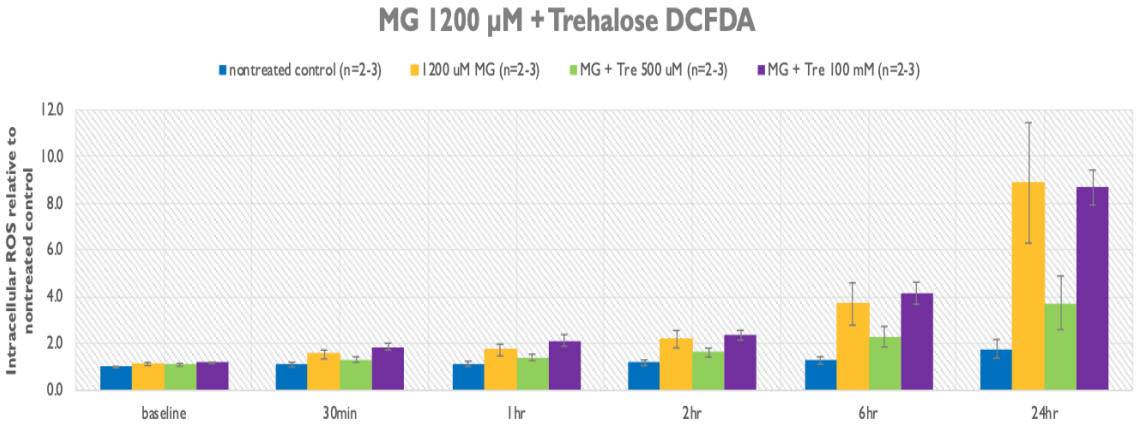

Figure 3.14 Trehalose cotreatment with 1200 µM MG effect on intracellular ROS by DCFDA assay. Trehalose showed a trend to reduce intracellular ROS levels only at lower concentration when compared to MG. Cardiomyoblast intracellular ROS was measured from baseline – 24 hours after adding 1200 µM MG with increasing concentrations of trehalose.

3.3.3 Reactive Oxygen Species of H9c2 Cells exposed to H_2O_2 and Rapamycin

The effects of rapamycin (100 nM - 20 µM, n=2-3) co-treated with H_2O_2 (600 µM) on ROS levels in H9c2 cells are shown in **Figure 3.15**. H_2O_2 600 µM started to increase intracellular ROS levels at 30 min and continued to raise it through 24 hours when compared to nontreated control. By contrast, rapamycin (100 nM and 20 µM) showed a trend to reduce H_2O_2-induced increase in ROS from 30 min to 24 hours. The reduction was dose dependent. At 24 hours, rapamycin (100 nM and 20 µM) reduced intracellular ROS levels to 27.32 ± 5.29 and 18.18 ± 5.19, respectively, when compared to 600 µM H_2O_2 alone.

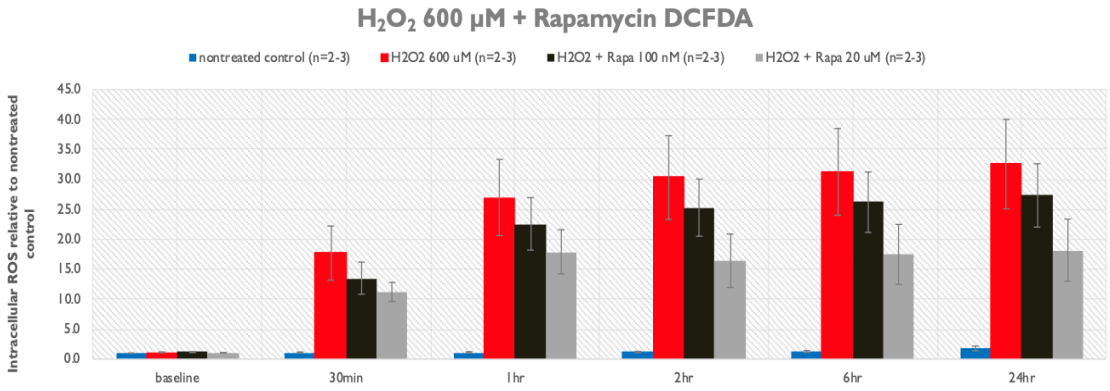

Figure 3.15 Rapamycin cotreatment with 600 µM H_2O_2 effect on intracellular ROS by DCFDA assay. Rapamycin showed a trend to reduce intracellular ROS levels when compared to H_2O_2. Cardiomyoblast intracellular ROS was measured from baseline – 24 hours after adding 600 µM H_2O_2 with increasing concentrations of rapamycin.

3.3.4 Reactive Oxygen Species of H9c2 Cells exposed to MG and Rapamycin

The effects of rapamycin (100 nM - 20 µM, n=2-3) co-treated with MG (1200 µM) on ROS levels in H9c2 cells are shown in **Figure 3.16**. MG (1200 µM) started to increase intracellular ROS levels at 2 hour and continued to raise it through 24 hours when compared to nontreated control. By contrast, rapamycin (100 nM and 20 µM) showed a trend to reduce MG-induced increase in ROS from 6 to 24 hours. At 24 hours, in the presence of MG (1200 µM), rapamycin (100 nM and 20 µM) increased intracellular ROS levels 6.29 ± 1.69-fold and 6.80 ± 1.93-fold, respectively, when compared to nontreated control, whereas MG (1200 µM) alone increased intracellular ROS levels 8.88 ± 2.58 times that of the nontreated control.

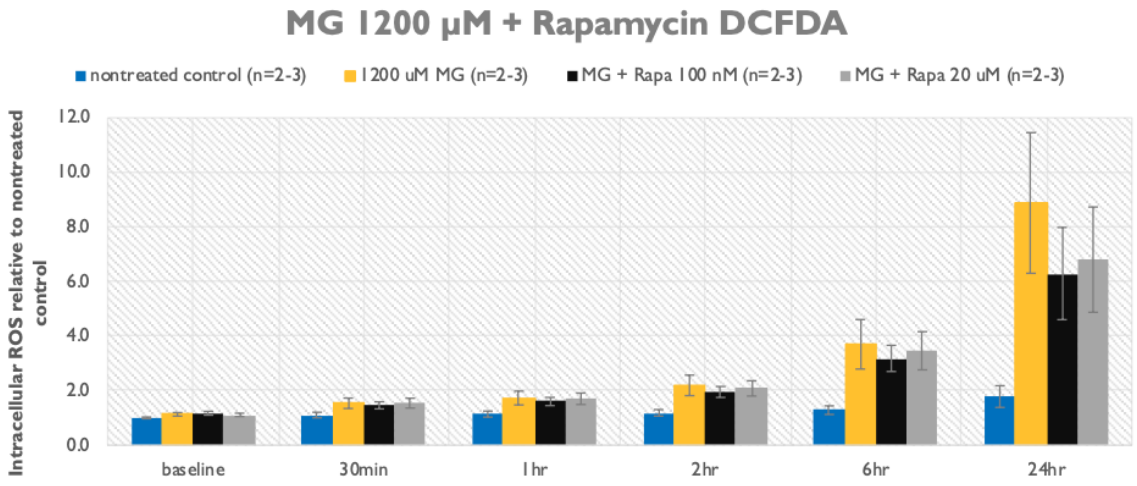

Figure 3.16 Rapamycin cotreatment with 1200 µM MG effect on intracellular ROS by DCFDA assay. Rapamycin showed a trend to reduce intracellular ROS levels at 24 hours when compared to MG. Cardiomyoblast intracellular ROS was measured from baseline – 24 hours after adding 1200 µM MG with increasing concentrations of rapamycin.

3.3.5 Reactive Oxygen Species of H9c2 Cells exposed to H_2O_2 and 3-methyladenine

The effects of 3-methyladenine (50 µM - 10,000 µM, n=2-3) co-treated with H_2O_2 (600 µM) on ROS levels in H9c2 cells are shown in **Figure 3.17**. H_2O_2 600 µM started to increase intracellular ROS levels at 30 min and continued to raise them through 24 hours when compared to nontreated control ROS levels. The lower dose of 3-MA (50 µM) showed a slight trend to reduce H_2O_2-induced increase in ROS from 30 min to 24 hours based on the preliminary data. By contrast, the higher dose 3-MA (10,000 µM) showed a slight increase in ROS from 30 min to 24 hours. At 24 hours, the ROS levels were increased 32.54 ± 7.47, 29.92 ± 7.21, and 34.32 ± 3.19-fold for 600 µM H_2O_2, 50 µM 3-MA, and 10,000 µM 3-MA, respectively. The higher the y-axis value, the greater the levels of intracellular ROS.

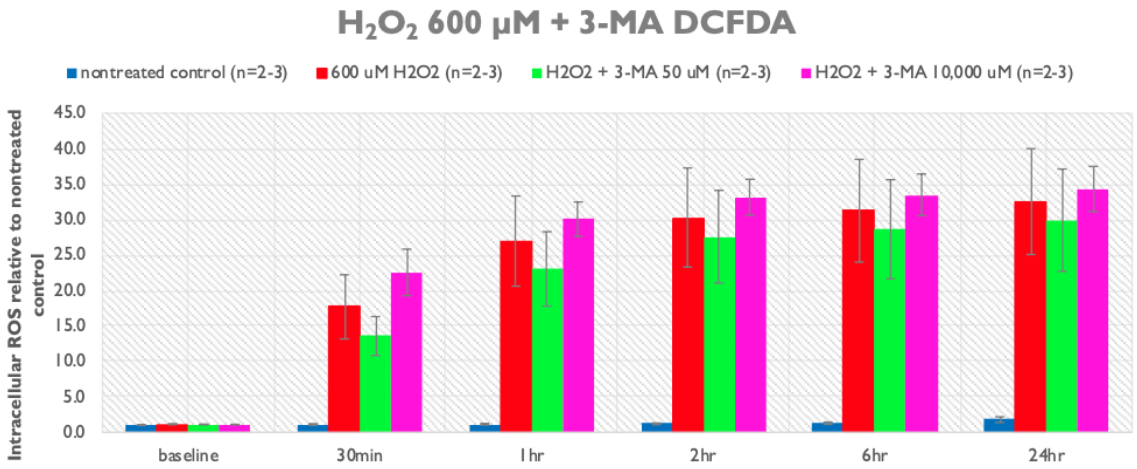

Figure 3.17 3-MA cotreatment with 600 µM H_2O_2 effect on intracellular ROS by DCFDA assay. 3-MA showed no obvious effects on intracellular ROS levels when compared to H_2O_2. Cardiomyoblast intracellular ROS was measured from baseline – 24 hours after adding 600 µM H_2O_2 with increasing concentrations of 3-MA.

3.3.6 Reactive Oxygen Species of H9c2 Cells exposed to MG and 3-methyladenine

The effects of 3-methyladenine (50 µM - 10,000 µM, n=2-3) co-treated with MG (1200 µM) on ROS levels of H9c2 cells are shown in **Figure 3.18**. MG (1200 µM) started to increase intracellular ROS levels at 2 hour and continued to raise them through 24 hours when compared to the nontreated control. By contrast, 3-MA (50 µM - 10,000 µM) showed slight reduction in MG-induced ROS increase only at 24 hours, based on the preliminary data. The ROS levels were increased 8.88 ± 2.58, 7.55 ± 1.38, and 6.97 ± 1.56-fold for 1200 µM MG, 50 µM 3-MA cotreatment, and 10,000 µM 3-MA cotreatment, respectively, at 24 hours.

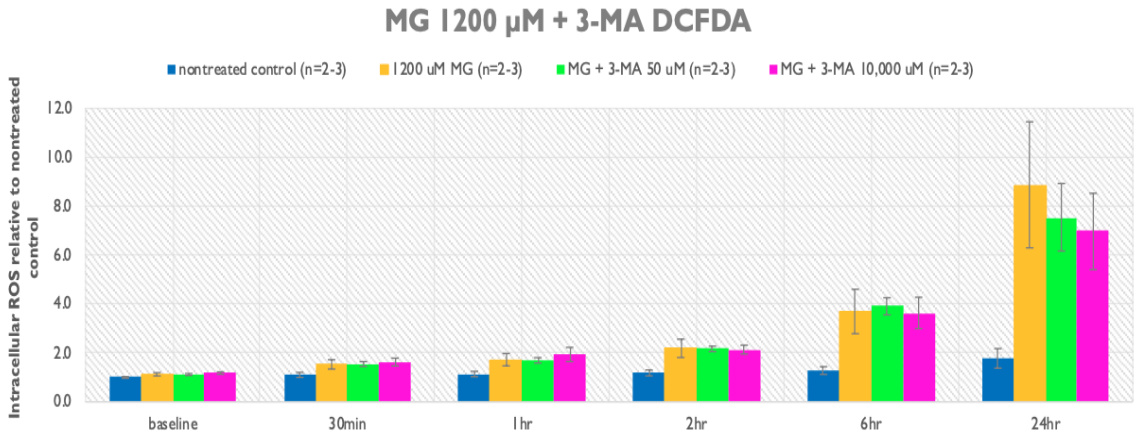

Figure 3.18 3-MA cotreatment with 1200 µM MG effect on intracellular ROS by DCFDA assay. 3-MA showed slight reduction of intracellular ROS levels at 24 hours when compared to MG. Cardiomyoblast intracellular ROS was measured from baseline – 24 hours after adding 1200 µM MG with increasing concentrations of 3-MA.

3.4 The effects of H_2O_2 and MG on Autophagy Rate

3.4.1 Effects of H_2O_2 on autophagy rate

The effects of H_2O_2 (100 µM - 600 µM, n=2) on autophagy are shown in **Figure 3.19**. The greater the y-axis value, the greater the number of autophagosomes present in the cell, therefore indicating higher levels of autophagy occurring. The preliminary data showed that H_2O_2 increased autophagy in a dose dependent manner only at 1 and 4 hours. Moreover, the same dose of H_2O_2 induced higher autophagy levels at 1 hour compared to 4 hours. By contrast, at 24 hours, lower doses (100 µM and 300 µM) showed a similar autophagy level increase as 1 hour whereas higher dose ranges (500 µM and 600 µM) reduced autophagy almost to the basal autophagy level. At 1, 4, and 24 hours, 300 µM H_2O_2 increased autophagy 1.72 ± 0.59, 1.14 ± 0.14, and 1.76 ± 0.63-fold, respectively, when compared to the negative control. By contrast, 600 µM H_2O_2 increased the

autophagy rate 2.30 ± 1.31, 1.96 ± 0.89, and 1.07 ± 0.26-fold, respectively, when compared to the negative control.

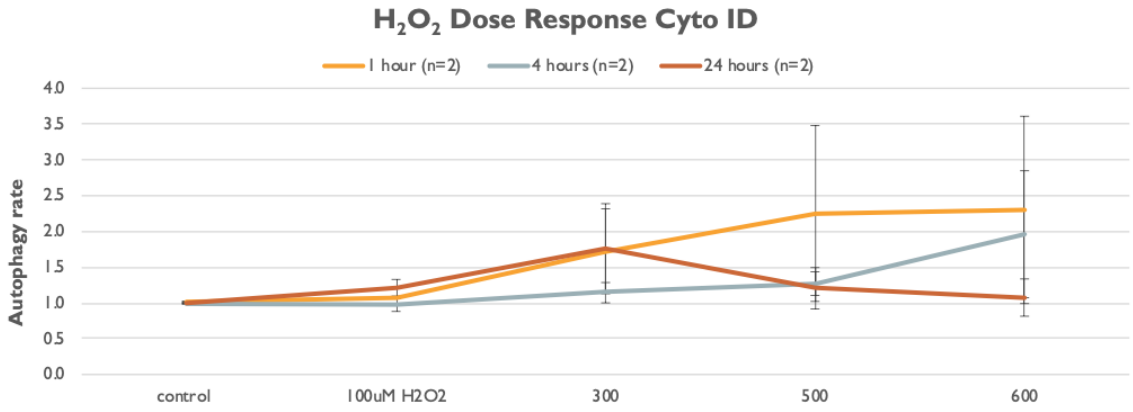

Figure 3.19 H_2O_2 dose response on autophagy rate by Cyto ID assay. H_2O_2 dose-dependently increased autophagy level at 1 and 4 hours but not at 24 hours. Autophagy level was measured at 1, 4, and 24 hours after adding increasing concentrations of H_2O_2 to the cardiomyoblasts.

3.4.2 Effects of MG on autophagy rate

The effects of MG (400 μM - 1200 μM, n=2) on autophagy are shown in **Figure 3.20**. The greater the y-axis value, the greater the number of autophagosomes present in the cell, therefore indicating higher levels of autophagy occurring. The preliminary data showed that MG (400 μM - 1200 μM) increased autophagy in a dose- and time-dependent manner only at 1and 24 hours when compared to the nontreated control. By contrast, there was no change in autophagy rate by MG at 4 hours. At 24 hours, autophagy was increased 1.39 ± 0.15, 1.84 ± 0.68, and 1.69 ± 0.75-fold for 400 μM MG, 800 μM MG, and 1200 μM MG, respectively, when compared to the nontreated control.

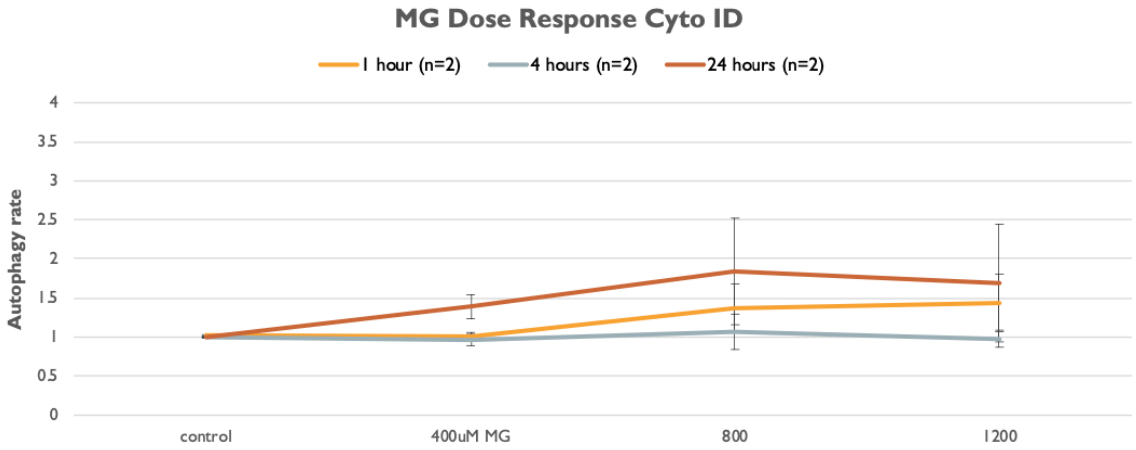

Figure 3.20 MG dose response on autophagy rate by Cyto ID assay. MG increased autophagy level in a dose-dependent and time-dependent manner. Autophagy level was measured at 1, 4, and 24 hours after adding increasing concentrations of MG to the cardiomyoblasts.

DISCUSSION

Our studies showed that both H_2O_2 and MG reduce H9c2 cell viability in a dose-dependent manner. The reduction of cell viability correlated with increased intracellular ROS levels. Also, preliminary Cyto ID data suggests that autophagy was induced by H_2O_2 or MG. The autophagy enhancing drug trehalose showed to have protective effects on H9c2 cells exposed to H_2O_2 at 24 hours only. However, trehalose protected cells against MG-induced cell damage at 1, 4, and 24 hours. Rapamycin, another autophagy enhancer, did not provide protection against H_2O_2. By contrast, only the low dose of rapamycin (100 nM) at 4 hours slightly increased the cell viability against MG-induced cell damage. Both trehalose and rapamycin reduced intracellular ROS levels induced by H_2O_2 or MG. By contrast, 3-MA, an autophagy inhibitor, also did not provide any increased cell viability to H9c2 cells exposed to H_2O_2. However, 3-MA did show protection against MG at 1 and 4 hours, but not 24 hours. 3-MA did not show any reduction in intracellular ROS levels induced by H_2O_2 or MG.

4.1 H_2O_2 and MG induced H9c2 cell damage and autophagy changes

4.1.1 H_2O_2 caused cell damage and autophagy rate increased

H_2O_2 often causes cell damage by forming ROS in the cell's mitochondria, therefore increasing the level of intracellular ROS. This occurs when superoxide species form in the mitochondria when free electrons escape the ETC and combine with O_2 forming superoxide. Superoxide dismutase then converts superoxide to H_2O_2. H_2O_2 would then normally be converted into H_2O by catalase; however, under disease

conditions H_2O_2 is produced at a faster rate than catalase can convert it to water. H_2O_2 is then converted to hydroxyl radicals, which are very reactive molecules; they can interact with cellular DNA, lipids, and proteins disrupting normal cellular function (Phaniendra et al, 2015). The CCK and calcein stain testing we conducted resulted in H_2O_2 reducing H9c2 cell viability in a dose-dependent manner from 100 μM – 600 μM with 600 μM H_2O_2 decreasing cell viability to about 60-80% compared to the nontreated control. The results we found were consistent with a study by Chen et al, 2000, which showed that H_2O_2 (200 – 600 μM) increased the percentage of dead H9c2 cells in a dose-dependent manner. The study determined that at 24 hours, 600 μM H_2O_2 increased the percentage of dead cells to 60% compared to the nontreated control (Chen et al, 2000).

As previously mentioned, H_2O_2 damages the cell leading to a decrease in cell viability by increasing intracellular ROS that damage important cellular DNA and proteins. In our experimentation we found that H_2O_2 increased intracellular ROS in a dose- and time-dependent manner, which was measured using DCFDA assay. Our study was confirmed by a study done by Amri et al, 2017 which tested how neuroglobin protected astroglial cells from oxidative stress caused by H_2O_2. Their study found that when astroglial cells were exposed to H_2O_2 (50 – 300 μM), the cellular ROS levels increased as the dose of H_2O_2 increased as well as when the exposure time to H_2O_2 increased (Amri et al, 2017).

Cells under oxidative stress through the increase of H_2O_2 will undergo the process of autophagy to try to decrease the damage of important cellular macromolecules caused by intracellular ROS. A vacuole called an autophagosome is formed by the signaling of Ulk-1 under oxidative stress. The autophagosome collects damaged proteins, lipids,

DNA, etc. and then fuses with a lysosome forming an autolysosome. The autolysosome then breaks down the damaged components into their monomeric form for the cell to be able to recycle and rebuild new functioning DNA, proteins, and lipids (Glick et al, 2010). H_2O_2 induces autophagy in cells by activation of AMP-activated protein kinase (AMPK). AMPK regulates the cell when the cell is under stress conditions; AMPK activation is necessary in order for the cell to survive. AMPK induces autophagy in cells by directly phosphorylating Ulk-1, which then activates Ulk-1 and signals for the autophagosome to form (She et al, 2014 and Viollet et al, 2011). A study done by She et al, 2014 experimented how AMPK protects against H_2O_2-induced apoptosis in osteoblasts. By use of Western blotting for AMPK, She found that AMPK activation depended on dose of H_2O_2 (100 – 250 μM) and exposure time (0 – 2 hours) to H_2O_2 (She et al, 2014).

In our study, we examined the levels of autophagy in H9c2 cells exposed to H_2O_2 by using Cyto ID, which detects autophagy levels in viable cells by determining the number of autophagosomes present. For H9c2 cells exposed to 100 – 600 μM H_2O_2, we saw that the rate of autophagy increased in a dose-dependent manner for 1 and 4 hours. For 24 hours we saw that the peak autophagy rate occurred at 300 μM H_2O_2, then the rate decreased from 500 – 600 μM. Our results of H_2O_2 increasing the rate of autophagy was correlated with a study done by Danyoung Li and Jingcheng Yu that studied how autophagy appeases oxidative stress in Mc3T3-E1 osteoblasts. In their study they determined that H_2O_2 (0 mM – 1mM) increased the rate of autophagy. Western blot results showed that as the dose of H_2O_2 was increased, the expression of autophagy-related proteins Beclin-1 and LC3II were increased in the osteoblasts, and MDC staining determined that the number of autophagosomes increased as the concentration of H_2O_2

increased (Li et al, 2017). We suggest that 24 hours has a slight decrease in the rate of autophagy after 300 μM H_2O_2 because at 500 - 600 μM H_2O_2, 60-80% of the cells are already dead compared to the nontreated control, based on the CCK data, and the Cyto ID analysis measures autophagy in viable cells only. If there are less viable cells at 24 hours, this will in turn display a lower level of autophagy occurring. Autophagy has also been shown to be known as a double-edged sword meaning that autophagy is capable of helping the cell under times of stress but can also damage the cell if there is too much stress. A study by Eileen White and Robert DiPaola discussed how autophagy is a double-edged sword in cancer and can be both beneficial and detrimental to cells. The study found that over stimulating autophagy by over-expressing Beclin-1 suppressed tumorigenesis. Over stimulation of autophagy occurs if the cell is under excessive oxidative stress; the cell then over-consumes DNA, lipids, and proteins providing more harm to the cell than benefit (White and DiPaola, 2009).

4.1.2 MG caused cell damage and autophagy rate increased

MG causes cell damage by increasing dicarbonyl stress. MG is a glycating agent that is formed as a by-product of metabolic processes, such as glycolysis. MG forms advanced glycation end products that can react with proteins and other cellular components, therefore altering and decreasing their function inducing cellular damage (Allaman et al, 2015 and Thornalley, 2008). Our CCK and calcein stain assays with MG showed that as the concentration of MG increases (400 μM – 1200 μM), cell viability decreases. 1200 μM MG decreased cell viability to about 60-80% compared to the nontreated control. Our study was complemented by a study performed by Seo et al, 2014. Seo actually performed the experimentation on HepG2 liver cells and with higher

doses of MG than in our study, but the results were similar in that MG dose-dependently decreased HepG2 cell viability. In their study, cell viability was assessed under MG concentrations of 1 mM – 10 mM, and 10 mM MG decreased cell viability the most when compared to the nontreated control (Seo et al, 2014).

We speculate the reason cell viability decreases is because MG raises the level of dicarbonyl stress in the cell by increasing intracellular ROS over physiological levels, which causes an increase in damage to the cell's DNA, lipids, and proteins. MG can cause increases in intracellular ROS by disrupting mitochondrial proteins that function as antioxidant mechanisms. For example, the advanced glycation end products formed from MG disrupt the function of superoxide dismutase and reduce the activity of catalase, both of which are important antioxidant enzymes (Desai et al, 2010 and Chen et al, 2018). In our study we used DCFDA assay, which measures intracellular ROS and determined that MG increased intracellular ROS in a dose- and time- dependent manner. Compared to a nontreated control, as the concentration of MG increased from 400 μM – 1200 μM, the level of ROS increased. As mentioned before, Seo, performed their experimentation on HepG2 liver cells and with higher doses of MG than in our study. Their study also examined intracellular ROS levels using DCFDA. Even though their study experimented with higher doses of MG (1 mM – 10 mM), their DCFDA results depicted higher ROS levels as the dose of MG was increased. However, their study did not include the factor of time since their DCFDA data was all collected at 12 hours (Seo et al, 2014).

Due to the increased damage caused by MG increasing intracellular ROS, the autophagy rate will also be increased. MG induces autophagy in a similar manner that H_2O_2 increases the rate of autophagy, by increasing AMPK activation (Dafre et al, 2017).

A study done by Dafre et al, 2017 tested how MG-induced activation led to autophagy degradation of thioredoxin 1 and glyoxalase 2 in HT22 nerve cells. Their results showed that AMPK phosphorylation occurred by MG in a dose- (0 - 0.5 mM) and time- (1 – 18 hours) dependent manner. Activation of AMPK resulted in inactivation of mTOR and therefore increased autophagy by phosphorylation of Ulk-1 (Dafre et al, 2017). In our study, using Cyto ID we found that the rate of autophagy increases as the concentration of MG (400 – 1200 μM) increases at 1 and 24 hours. Interestingly, 4 hours seems to not have that much of an increase in autophagy compared to the nontreated control. We actually do not have a firm explanation for why this has occurred; more Cyto ID testing is required. We can see based on the DCFDA data of MG that it does take time to produce sufficient ROS levels in the cardiomyocytes, which is why we see 24 hours containing the highest rate of autophagy, but why 1 hour shows a higher rate of autophagy than 4 hours is not understood as of yet. However, we do know based on previous studies that MG does increase the rate of autophagy. A study by Yo-Chen Chang et al, 2015 tested how MG increased the rate of autophagy and suppressed cell division in human retinal pigment ARPE-19 epithelial cells. This study also came to conclude that MG increased the autophagy flux in the epithelial cells. When treated with MG (125 μM – 500 μM) Western blot showed that the epithelial cells showed an increased production of LC3II as the dose of MG increased. The experiment also looked at 250 μM MG at different times (0 – 24 hours) to determine if exposure time would increase the autophagy flux. Results showed that exposure to 250 μM MG increased the presence of LC3II as exposure time was increased, based on Western blot analysis (Chang et al, 2015).

4.2 Trehalose effects on cell viability and intracellular ROS in H9c2 cells exposed to H_2O_2 or MG

Trehalose is a disaccharide sugar not found in mammals (Hosseinpour-Moghaddam et al, 2018), but when introduced to mammals trehalose works as an autophagy enhancer. Trehalose enhances autophagy by inhibiting glucose and fructose from entering the cell; this induces a starvation-like state in the cell which then signals for autophagy to occur by stimulation of AMPK and activation of the Ulk-1 complex (Mardones, Rubinsztein, and Hetz, 2016).

Trehalose also is capable of reducing intracellular ROS levels by upregulating the Kelch-like ECH-associated protein 1- NF-E2 related factor 2 (Keap1-Nrf2) pathway (Mizunoe et al, 2018). Nrf2 is important in regulating antioxidant mechanisms in the cell to reduce ROS. Nrf2 controls the gene expression of enzymes involved in eliminating ROS. When a cell is under oxidative stress conditions, the cell alters metabolism and gene expression to maintain redox homeostasis; it does so by activating Nrf2 as well as other stress response pathways. Keap1 acts as a regulatory protein of Nrf2; under unstressed conditions, Keap1 ubiquitinates Nrf2 (Kasai et al, 2020). A study by Mizunoe et al, 2018 showed how trehalose protects against oxidative stress by regulation of Keap1-Nrf2. Results showed that trehalose upregulated Nrf2 genes and therefore decreased intracellular ROS via antioxidant mechanism. In their study, when compared to paraquat, which is an experimental ROS generator, trehalose (50 mM) reduced intracellular ROS when analyzed by DCFDA (Mizunoe et al, 2018).

In our study when trehalose (500 μM – 100 mM) was introduced to cells exposed to H_2O_2 (600 μM) we found that cell viability increased at 24 hours; however, at 1 and 4

hours there did not seem to be any increase in cell viability compared to H_2O_2 (600 μM). We also determined that trehalose decreased intracellular ROS in H9c2 cells exposed to H_2O_2 (600 μM) compared to H9c2 cells exposed to H_2O_2 (600 μM) alone. Our DCFDA analysis was only done two times, so statistical analysis was not able to conclude any significance; this experimentation will need to be done a few more times in the future. However, based on the experiments we have done, we do see that the lower dose of trehalose (500 μM) and the higher dose of trehalose (100 mM) both decrease intracellular ROS compared to the positive control (600 μM H_2O_2).

In our experiment of trehalose with MG, we found that trehalose (500 μM – 100 mM) increased cell viability in H9c2 exposed to MG (1200 μM) at 1, 4, and 24 hours compared to the positive control (MG 1200 μM). The highest increase in cell viability occurred at 24 hours with exposure to 5 mM trehalose; the cell viability then decreased from 10 – 100 mM trehalose. It's possible that the decrease in cell viability from 10 – 100 mM trehalose at 24 hours occurs because if a cell is under stress and too much autophagy occurs, then there is too much self-consumption further damaging the cell leading to cell death as mentioned before with autophagy being a double-edged sword. DCFDA data for trehalose with MG shows that the lower dose of trehalose (500 μM) decreases intracellular ROS more than the higher dose of trehalose (100 mM) when compared to the positive control. Our DCFDA data shows that at 24 hours 100 mM trehalose cotreatment has intracellular ROS levels similar to the positive control therefore the H9c2 cells are under sufficient stress, so enhancing autophagy could be overkill for the cell causing the decrease in cell viability.

Our study on trehalose with H_2O_2 increasing cell viability was correlated with a study done by Darabi et al, 2018 who experimented on how trehalose activates autophagy to prevent H_2O_2-induced cell death in bone marrow stromal cells. The study found that after 8 hours H_2O_2 decreased cell viability to 82%, 72%, 49%, and 39% of the control after receiving 50, 100, 200, and 400 μM of H_2O_2, respectively. However, with pretreatment of trehalose 3% for 2 hours before exposure to H_2O_2, the cells increased cell viability to 92%, 84%, 75%, and 48% of the control after receiving 50, 100, 200, and 400 μM of H_2O_2, respectively (Darabi et al, 2018). Also, our study of trehalose decreasing intracellular ROS compared to a study done by Gao et al, 2018, which experimented on how trehalose reduced damage caused by H_2O_2 in dopaminergic SH-SY5Y cells. Using DCFH-DA Gao was able to determine how trehalose affected the level of ROS produced by H_2O_2 in the SH-SY5Y cells. To test this, the cells were pretreated with 5mM trehalose for 48 hours, then the cells were incubated with H_2O_2 (250 μM or 500 μM) for 6 hours. The results showed that trehalose inhibited H_2O_2-induced increases of ROS in SH-SY5Y cells, therefore maintaining the proper function of antioxidant enzymes such as superoxide dismutase and catalase (Gao et al, 2018).

4.3 Rapamycin effects on cell viability and intracellular ROS in H9c2 cells exposed to H_2O_2 or MG

Rapamycin is a natural drug that possesses strong antifungal and immunosuppressant properties. It is an autophagy enhancer that works by binding FKBP12 protein and forming a complex that inhibits mTOR, which is a regulatory protein of autophagy that inhibits the process of autophagy. Rapamycin inhibits mTOR and increases Ulk-1 activity, which therefore signals for autophagy to proceed (Yang et

al, 2013). There are two complexes of mTOR, mTORC1 and mTORC2. Rapamycin directly inhibits mTORC1; however, rapamycin needs chronic administration to inhibit mTORC2 in some cell lines. The ability of a cell to respond to treatment of rapamycin depends on the expression of FKBP12; reduction of FKBP12 levels in cell lines can block rapamycin's ability to inhibit mTORC1 and mTORC2 (Schreiber et al,2015).

Our study showed that rapamycin (100 nM – 20 μM) did not increase cell viability compared to the positive control (600 μM H_2O_2). Looking at our DCFDA data we saw that both the low dose of rapamycin (100 nM) and the high dose of rapamycin (20 μM) decreased intracellular ROS compared to the positive control (600 μM H_2O_2), so we expected to see that cell viability would also increases since the oxidative stress is reduced in those cells. Our study was countered by a study by Gao et al, 2020 that experimented with the effects of rapamycin on rat H9c2 cardiomyocytes with chronic heart failure. Results of this study found that rapamycin improved cardiomyocyte function by promoting autophagy in rats with heart failure. Using Western blotting, Gao found that rapamycin inhibited caspase-3 expression, which is important for the process of apoptosis, and upregulated important autophagy proteins, LC3 II, Beclin-1, and p62. Gao also explored the mechanism by which rapamycin inhibits mTOR in rats with chronic HF. Gao looked at the activities of mTORC1 and mTORC2 as denoted by phosphorylation of their targets downstream S6 and Akt, respectively. Results showed that rapamycin inhibited the phosphorylation of S6 and Akt therefore promoting autophagy (Gao et al, 2020). However, the concentration of rapamycin (1.4 mg-kg-day) and the time period of administration (8-12 weeks) were higher and longer than the concentrations and times used in our study.

Another study by Ding et al, 2017 used concentrations of rapamycin similar to what we used for our experimentation and found that rapamycin is actually detrimental to cardiac myocytes in rats. Results showed that rapamycin (1 nM – 100 μM) reduced cell survival rate compared to a nontreated control. In this study, rapamycin's ability to induce autophagy caused cardiac myocyte death (Ding et al, 2017). The findings of this study seem to support our own data, which indicate that rapamycin (100 nM – 20 μM) did not increase cell viability in cells treated with 600 μM H_2O_2. However, because of the conflicting data found when comparing research studies, as well as the conflicting data in our own studies, it is clear that more experiments will need to be done with rapamycin on H9c2 cell viability and intracellular ROS levels.

In our experiment of rapamycin (100 nM – 20 μM) with H9c2 cells exposed to MG (1200 μM), we can see that there is only one dose of rapamycin (100 nM) at 4 hours that shows a significant increase in cell viability compared to the positive control (1200 μM MG). Other than the one dose, rapamycin does not seem to increase cell viability in the cardiomyocytes. The DCFDA data also does not show much reduction in intracellular ROS levels when either 100 nM or 20 μM rapamycin are added compared to 1200 μM MG.

Rapamycin has been found to increase glycolytic intermediates, such as G6P and DHAP among others (Tucci et al, 2013). An increase in DHAP and GP6 would mean an increase in MG formation, which could contribute to the lack of increased cell viability by rapamycin. Tucci et al, 2013 also tested the effects of rapamycin on glutathione, which is an important antioxidant path in cells. Results showed a possible increase in glutathione when rapamycin was introduced into rat fibroblast cells (Tucci et al, 2013).

Another study by Shin et al, 2011 did find that rapamycin protected human corneal endothelial cells by reducing intracellular ROS by increasing intracellular glutathione (Shin et al, 2011). This could explain why in our study we saw decreases in intracellular ROS compared to our positive controls; however, more experimentation with rapamycin will need to be done.

4.4 3-methyladenine effects on cell viability and intracellular ROS in H9c2 cells exposed to H_2O_2 or MG

3-methyladenine is an autophagy inhibitor that works by inhibiting class III PI3K complex. This then blocks PI3K from forming, which would normally recruit Atg proteins for the initiation of autophagy (Wu et al, 2010). As we expected, our study shows that introducing 3-MA with H_2O_2 (600 μM), 3-MA (50 – 10,000 μM) does not provide any increased cell viability compared to the positive control (600 μM H_2O_2), and DCFDA data shows that 3-MA does not decrease intracellular ROS. The higher dose of 3-MA (10,000 μM) increases intracellular ROS slightly higher than the positive control. When introducing 3-MA with MG (1200 μM) we found that 3-MA (50 – 10,000 μM) increased cell viability at 1 and 4 hours compared to the positive control (1200 μM MG); 24 hours did not show any increased cell viability. The DCFDA data showed that from baseline – 6 hours 3-MA (50 μM or 10,000 μM) did not reduce intracellular ROS compared to the positive control; at 24 hours there was a slight decrease in intracellular ROS by both the low dose and high dose of 3-MA compared to the positive control.

We speculate that 3-MA provides protection for MG but not for H_2O_2 because 3-MA has been shown to have a dual role as demonstrated by Wu et al, 2010. Wu et al, 2010 has shown that 3-MA induced autophagy under nutrient-rich conditions. 3-MA

works by inhibiting PI3K, which of there are two classes of: class I PI3K and class III PI3K. 3-MA targets both of these without discrimination. Inhibition of class III PI3K will prevent autophagy from occurring; however, inhibition of class I PI3K promotes autophagy. Under nutrient-rich conditions, 3-MA seemed to inhibit class I PI3K, while the function of class III PI3K was preserved therefore promoting autophagy (Wu et al, 2010). In our study we see that 600 μM H_2O_2 increases intracellular ROS rather quickly, therefore causing stress in the cell and possibly inducing a nutrient-deficient state rapidly. As discussed in the study by Wu, in a nutrient-deficient state, 3-MA does not induce autophagy, which is why we believe we do not see an increase in cell viability in our study. Meanwhile, MG does not induce oxidative stress as quickly as H_2O_2; our DCFDA data shows that the highest levels of intracellular ROS occur at 24 hours, so we speculate that at 1 and 4 hours there is still viable nutrients that the cell can receive, therefore allowing 3-MA to inhibit class I PI3K instead of class III PI3K consequently promoting autophagy. Then at 24 hours, enough time has passed for MG to induce oxidative stress and starve the cell of sufficient nutrients preventing 3-MA from promoting autophagy and increasing cell viability.

A study by Shin et al, 2014 correlates with our study's findings that 3-MA increases intracellular ROS levels. Shin did a study on the relationship between ROS and autophagy in mouse blastocysts. Results of the study found that daily injections of 5 mM 3-MA increased intracellular ROS levels in the blastocysts (Shin et al, 2014).

4.5 Future Studies

In the future it will be required for us to do more DCFDA testing since all of our DCFDA experiments were run just 2-3 times. Cyto Id analysis for measuring autophagy

will also need to be run again since that experimentation was also done only twice. For Cyto ID we think it will be beneficial in the future to examine the autophagy rates with the autophagy regulatory drugs we used, since we had only done dose response Cyto ID experimentation on H_2O_2 and MG. More experimentations will need to be done on rapamycin to evaluate more on why ROS was decreased, but cell viability did not decrease. We also find it important to choose another autophagy inhibitor besides 3-MA to test the effects of cell viability and intracellular ROS levels, since 3-MA has a dual role; by picking an inhibitor that does not seem to have a dual role, such as chloroquine, we can perhaps get a better understanding of the role of an autophagy inhibitor and also use chloroquine as a comparison to 3-MA.

4.6 Conclusion

In conclusion we determined that H_2O_2 and MG decrease cell viability and increase intracellular ROS of H9c2 cells in a dose-dependent manner. The autophagy enhancing drug trehalose increased cell viability of H9c2 cells exposed to H_2O_2 at 24 hours, and increased cell viability of cells exposed to MG at 1, 4, and 24 hours by decreasing intracellular ROS. Rapamycin, another autophagy enhancer, which although decreased intracellular ROS, did not increase cell viability in cells exposed to H_2O_2, and for cells exposed to MG, only the low dose of rapamycin at 4 hours provided an increase in cell viability. 3-methyladenine, an autophagy inhibitor, did not provide decreases in intracellular ROS and therefore did not increase cell viability of H9c2 cells exposed to H_2O_2. For H9c2 cells exposed to MG, we saw 3-MA increased cell viability at 1 and 4 hours although intracellular ROS was not decreased. For autophagy rate of H_2O_2, we saw the autophagy rate increased in dose-dependent manner at 1 and 4 hours, but at 24 hours,

the autophagy rate peaked at 300 μM, then decreased at doses higher than that. For MG, the autophagy rate increased in a dose-dependent manner at 1 and 24 hours, but at 4 hours, there was no increase in the rate.

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